GUILHERME PUGLIESI

SECREÇÃO DE PROSTAGLANDINAS ANTES, DURANTE E APÓS A LUTEÓLISE EM BOVINOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Zootecnia, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 16 de maio de 2012.

_____	_____
Prof. Ciro Alexandre Alves Torres (Coorientador)	Prof. Álan Maia Borges
_____	_____
Prof. Eduardo Paulino da Costa	Prof. José Domingos Guimarães

_____
Prof. Giovanni Ribeiro de Carvalho
(Orientador)

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Dedico

"O êxito começa no momento exato em que o homem decide o que quer e começa a trabalhar para o conseguir"

(Roberto Shinyashiki)

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BIOGRAFIA

GUILHERME PUGLIESI, filho de Dorival Pugliesi e Conceição Aparecida Pereira Pugliesi, nasceu na cidade de Franca, São Paulo, em 5 de agosto de 1984.

Em outubro de 2002, ingressou no curso de graduação em Medicina Veterinária na Universidade Federal de Minas Gerais.

Em agosto de 2007, iniciou o programa de pós-graduação do Departamento de Zootecnia da UFV, em nível de mestrado, concentrando seus estudos na área de Biotecnologia da Reprodução Animal.

Em Fevereiro de 2009, iniciou o programa de pós-graduação do Departamento de Zootecnia da UFV, em nível de doutorado, concentrando seus estudos na área de Fisiologia da Reprodução Animal.

Em Janeiro de 2010 até Julho de 2011, realizou estágio em doutoramento na University of Wisconsin, onde desenvolveu os estudos aqui descritos.

Em Maio de 2012, submeteu-se à defesa desta tese.

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RESUMO

PUGLIESI, Guilherme., D. Sc., Universidade Federal de Viçosa, Maio de 2012. **Secreção de prostaglandinas antes, durante e após a luteólise em bovinos.** Orientador: Giovanni Ribeiro de Carvalho. Coorientadores: Oliver Joseph Ginther e Ciro Alexandre Alves Torres.

O presente estudo foi elaborado com o objetivo de avaliar o papel da secreção de prostaglandina F2α (PGF2α) antes, durante e após o período luteolítico na regressão funcional e estrutural do corpo lúteo (CL) em novilhas; elucidar os efeitos do estradiol-17β (E2) em diferentes níveis fisiológicos na secreção de PGF2α e indução da luteólise; e estudar os efeitos da inibição das prostagladinas no período pós-luteolítico sobre o crescimento folicular e ovulação. Foram realizados quatro estudos sequenciais, sendo o primeiro estudo (**Estudo 1**) composto por dois experimentos e os demais estudos (**Estudos 2, 3** e **4**) compostos por um experimento cada. No **Estudo 1** objetivou-se avaliar os efeitos da inibição da síntese de PGF2α durante os períodos de pré-luteólise e luteólise, baseando-se na mensuração da concentração plasmática do metabólito da PGF2α (PGFM). Para isto, no **Experimento 1**, flunexine meglumine (FM) foi administrado em diferentes doses (0, 0,5, 1,0 ou 1,5 g) em novilhas (n = 4/grupo) no 16º dia pós-ovulação para se determinar a dose mínima eficaz para inibir a síntese de PGF2α no período esperado da luteólise espontânea. A dose de 1,5 g, que correspondeu em média 2,5 mg/kg de peso vivo, foi mais eficaz em reduzir as concentrações de PGFM comparada as demais doses. Assim, no **Experimento 2,** FM (2,5 mg/kg) foi administrado a cada 8 horas por 24 horas (primeiro tratamento =

Hora 0 no 15º dia pós-ovulação). Amostras de sangue foram colhidas em intervalos de 8 horas do 15º ao 18º dia pós-ovulação em um grupo veículo (controle) e um grupo tratado com FM (n = 16/grupo). Adicionalmente, amostras de sangue foram colhidas de hora em hora da Hora –2 a 28 em 10 novilhas de cada grupo. As novilhas foram subdivididas naquelas que estavam em pré-luteólise e luteólise na Hora 0 baseado nas concentrações plasmáticas de progesterona (P4) realizadas de 8 em 8 horas. As concentrações de PGFM foram reduzidas ($P < 0,05$) pelo tratamento com FM em cada subgrupo. Para o subgrupo pré-luteolítico, o primeiro decréscimo ($P < 0,05$) nas concentrações de P4 ocorreu, respectivamente, nas Horas 24 e 40 nos grupos veículo e FM. As concentrações de P4, 32 e 40 horas após o início da luteólise no subgrupo luteolítico foram maiores ($P < 0,05$) no grupo FM. As concentrações de PGFM no pico do pulso de PGFM no grupo FM foram maiores ($P < 0,05$) no subgrupo luteolítico do que no subgrupo pré-luteolítico. No segundo estudo (**Estudo 2**), objetivou-se estudar o efeito da concentração de E2 na proeminência dos pulsos de PGFM e sua relação com as concentrações de P4, hormônio luteinizante (LH), e fluxo sanguíneo luteal em novilhas em pré-luteólise. Para isto, uma única dose de 0 (veículo), 0,01, 0,05, ou 0,1 mg de E2 foi administrado (n=6/grupo) 14 dias pós-ovulação. Amostras de sangue foram colhidas e o fluxo sanguíneo luteal estimado de hora em hora por 10 horas logo após o tratamento. A dose de 0,05 mg resultou em um aumento intermediário e a dose de 0,1-mg promoveu um aumento mais pronunciado no pulso de PGFM, comparado com as doses de 0,0 e 0,01 mg. Todos os pulsos de PGFM foram agrupados e subdivididos em três diferentes categorias de proeminência (< 50, 50–150, e > 150 pg/mL no pico do pulso). Na categoria de 50–150, a concentração de P4 aumentou ($P < 0,05$) no pico do pulso de PGFM. Na categoria >150, a concentração de P4 reduziu ($P < 0,05$) no pico dos pulsos de PGFM, a de LH aumentou ($P < 0,05$) após uma hora do pico, e o fluxo sanguíneo luteal aparentemente reduziu ($P < 0,05$) 2 h após o pico de PGFM. Os resultados suportaram as seguintes hipóteses: 1) um aumento nas concentrações de E2 eleva a proeminência do pulso de PGFM, e 2) a maior proeminência do pulso de PGFM é associado com maior depressão de P4 no pico do pulso de PGFM. Adicionalmente, a dimensão do efeito da PGF2α no aumento das concentrações de LH e nas mudanças do fluxo sanguíneo luteal

dentro das horas de um pulso de PGFM foi positivamente relacionada com a proeminência do pulso de PGFM. No estudo subsequente (**Estudo 3**), objetivou-se elucidar os efeitos da indução sequencial de pulsos de PGFM pelo tratamento com E2 sobre a proeminência dos pulsos de PGFM e na indução da luteólise. Três tratamentos com veículo (n = 12) ou E2 (n = 12) nas doses de 0,05 mg ou 0,1 mg foram administrados em intervalos de 12 horas, iniciando-se no 15º dia pós-ovulação. Amostras de sangue foram colhidas a cada 12 horas entre o 13º e o 24º dia pós-ovulação, e de hora em hora por um período de 12 horas após o primeiro e o terceiro tratamentos. No dia 15 pós-ovulação, todas as novilhas estavam em fase de pré-luteólise e no dia 16 estavam em pré-luteólise no grupo administrado veículo (n =11) e em pré-luteólise (n = 4) ou luteólise (n = 8) no grupo tratado com E2. A concentração de PGFM no pico dos pulsos de PGFM induzidos durante o período de pré-luteólise no 15º dia pós-ovulação foi maior ($P < 0,04$) do que dos pulsos de PGFM pré-luteolíticos no 16º dia pós-ovulação. O intervalo da ovulação até o início da luteólise foi reduzido ($P < 0,05$) nas novilhas tratadas com E2 quando comparado as demais novilhas não tratadas (veículo). O pulso de PGFM induzido pelo E2 foi menos proeminente ($P < 0,008$) nas novilhas que apresentaram resurgência transitória das concentrações de P4 do que nas novilhas que apresentaram um decréscimo progressivo da P4. A hipótese que a exposição repetida ao E2 estimula o aumento da proeminência dos pulsos de PGFM não foi suportada. Ao contrário, a exposição repetida reduziu a proeminência dos pulsos de PGFM em contraste ao estímullo promovido após o primeiro tratamento. Estes resultados indicaram pela primeira vez que a redução na proeminência dos pulsos de PGF2α durante a luteólise pode resultar em resurgimento transitório na concentração de P4. No **Estudo 4**, objetivou-se avaliar os efeitos da inibição da síntese de prostaglandinas durante a pós-luteólise na regressão do CL, secreção de prolactina (PRL), e crescimento folicular e ovulação em novilhas. O início da pós-luteólise (P4 < 1 ng/mL) foi identificado e definido como 8 horas após a detecção pela ultrassonografia de 25% de redução na área (cm^2) do CL, e foi assim designado Hora 0. Foi administrado via intramuscular FM (2,5 mg/kg; n = 10) para inibir a secreção de PGF2α ou veículo (n = 9) nas Horas 0, 4, 8, 16, 24, 32, e 40. Foi realizado amostragem sanguínea e mensuração do CL e do folículo dominante a cada 8 horas iniciando no 14º dia pós-ovulação.

Amostras de sangue foram também colhidas de hora em hora por 24 horas iniciando na Hora 0 para detecção dos pulsos de PRL e PGFM. As concentrações nos pulsos de PGFM e PRL foram inferiores no grupo FM comparado ao grupo veículo. A concentração de PRL foi maior no pico do pulso de PGFM do que nas demais horas do pulso. Tanto a área do CL quanto a concentração de P4 não diferiram entre os grupos durante as Horas 0 a 48 (pós-luteólise). A ovulação ocorreu em nove de nove novilhas no grupo veículo e em três de 10 novilhas do grupo FM. Os folículos anovulatórios no grupo FM cresceram até 36,2 ± 2,9 mm, e a parede folicular se apresentou espessada em decorrência de aparente luteinização. A hipótese que a PGF2α estava envolvida na redução continua da secreção de P4 e na regressão estrutural do CL durante a pós-luteólise não foi suportada. As hipóteses que os pulsos de PGFM e PRL estavam temporariamente relacionados e que o tratamento com FM sistêmico induziu a formação de folículos anovulatórios foram suportadas.

ABSTRACT

PUGLIESI, Guilherme, D. Sc., Universidade Federal de Viçosa, May, 2012. **Role of prostaglandins secretion before, during, and after luteolysis in cattle.** Adviser: Giovanni Ribeiro de Carvalho. Co-advisers: Oliver Joseph Ginther and Ciro Alexandre Alves Torres.

The current study was done with the aim to evaluate the role of prostaglandin F2α (PGF2α) synthesis before, during, and afer the luteolytic period on functional and morphological corpus luteum (CL) regression; study the effects of estradiol-17β (E2) in diferrent physiological levels on PGF2α secretion and induction of luteolysis; and evaluate the effects of prostaglandin inhibition during postluteolysis on follicular growth and ovulation in heifers. Four sequential studies were done. The first (**Study 1**) was elaborated from two experiments, and the other three studies (**Studies 2, 3** and **4**) were compound by one experiment each. The **Study 1** aimed to evaluate the inhibition of the synthesis of prostaglandin F2α (PGF2α) during preluteolytic and luteolytic periods, based on plasma concentrations of a PGF2α metabolite (PGFM). In **Experiment 1**, flunixin meglumine (FM; 2.5 mg/kg) was given to heifers (n = 4/group) in diferrent doses (0, 0.5, 1.0, or 1.5 g) 16 days after ovulation to evaluate the minimal effective dose during the expected luteolytic period. The dose of 1.5 g (equivalent to 2.5 mg/kg bw) was the most effective. Therefore, in **Experiment 2** FM (2.5 mg/kg) was given to heifers at three 8-h intervals, 16 days after ovulation (first treatment = Hour 0). Blood samples were collected at 8-h intervals from 15 to 18 d postovulation in a vehicle (control) and FM group (n = 16/group). Hourly samples were collected from Hours –2 to 28 in 10 heifers

in each group. Heifers that were in preluteolysis or luteolysis at Hour 0 based on plasma progesterone (P4) concentrations at 8-h intervals were partitioned into preluteolytic and luteolytic subgroups. Concentration of PGFM was reduced ($P < 0.05$) by FM treatment in each subgroup. For the preluteolytic subgroup, the first decrease ($P < 0.05$) in P4 concentration after Hour 0 occurred at Hours 24 and 40 in the vehicle and FM groups, respectively. Plasma P4 concentrations 32 and 40 h after the beginning of luteolysis in the luteolytic subgroup were greater ($P < 0.05$) in the FM group. Concentration at the peak of a PGFM pulse in the FM group was greater ($P < 0.05$) in the luteolytic than in the preluteolytic subgroup. In the second study (**Study 2**), we aimed to evaluate the effect of E2 concentration on the prominence of PGFM pulses and the relationship between prominence and intrapulse concentration of P4, luinizing hormone (LH), and luteal blood flow were studied. A single dose of 0 (vehicle), 0.01, 0.05, or 0.1 mg of E2 was given (n=6/group) 14 days after ovulation. Blood samples were collected and luteal blood flow was evaluated hourly for 10 h after the treatment. The 0.05-mg dose increased and the 0.1 mg further increased the prominence of the induced PGFM pulse, compared to the 0.0-mg dose and the 0.01-mg dose. The PGFM pulses were subdivided into three different prominence categories (< 50, 50–150, and > 150 pg/mL at the peak). In the 50–150 category, P4 concentration increased ($P < 0.05$) between –2 h and 0 h (0 h = peak of PGFM pulse). In the >150 category, P4 decreased ($P < 0.05$) between –1 h and 0 h, LH increased ($P < 0.05$) at 1 h, and luteal blood flow apparently decreased ($P < 0.05$) at 2 h of the PGFM pulse. The novel results supported the following hypotheses: 1) an increase in E2 concentration increases the prominence of a PGFM pulse, and 2) greater prominence of a PGFM pulse is associated with a greater transient intrapulse depression of P4 at the peak of the PGFM pulse. In addition, the extent of the effect of PGF2α on the increase in LH and changes in blood flow within the hours of a PGFM pulse was related positively to the prominence of the PGFM pulse. In the subsequent study (**Study 3**), we aimed to elucidate the effects of sequential induction of PGFM pulses by E2 on prominence of PGFM pulses and P4 concentration. Three treatments of vehicle (n = 12) or E2 (n = 12) at doses of 0.05 mg or 0.1 mg were given at 12-h intervals beginning on day 15 postovulation. Blood samples were collected every 12 h from days 13 to 24 postovulation and hourly for 12 h after

the first and third treatments. On day 15, all heifers were in preluteolysis and on day 16 were in preluteolysis in the vehicle-treated heifers (n = 11) and either preluteolysis (n = 4) or luteolysis (n = 8) in the E2-treated heifers. Peak concentration of induced PGFM pulses during preluteolysis on day 15 was greater ($P < 0.04$) than for pulses during preluteolysis on day 16. The interval from ovulation to the beginning of luteolysis was shorter ($P < 0.04$) in the E2-treated heifers than in the vehicle-treated heifers. An E2-induced PGFM pulse was less prominent ($P < 0.008$) in heifers in temporal association with a transient resurgence in P4 than in heifers with a progressive P4 decrease. The hypothesis that repeated E2 exposure stimulates increasing prominence of PGFM pulses was not supported. Instead, repeated exposure reduced the prominence of PGFM pulses in contrast to the stimulation from the first E2 treatment. Results indicated for the first time that reduced prominence of a PGF2α pulse during luteolysis can lead to a transient resurgence in P4 concentration. The **Study 4** aimed to study the effects of prostaglandin inhibition during postluteolysis on CL regression, prolactin (PRL) secretion, and follicle growth and ovulation in heifers. The beginning of postluteolysis (P4 < 1 ng/mL) was targeted by using 8 h after ultrasonic detection of a 25% decrease in CL area (cm^2) and was designated Hour 0. FM (2.5 mg/kg; n = 10) to inhibit PGF2α secretion or vehicle (n = 9) were given intramuscularly at Hours 0, 4, 8, 16, 24, 32, and 40. Blood sampling and measurement of the CL and dominant follicle were done every 8 h beginning 14 days postovulation in each group. Blood samples for detection of pulses of PRL and pulses of PGFM were obtained every hour for 24 h beginning at Hour 0. Pulse concentrations of both PGFM and PRL were lower in the FM group than in the vehicle group. Concentration of PRL was greatest at the peak of a PGFM pulse. Neither CL area (cm^2) nor P4 concentration differed between groups during Hours 0 to 48 (postluteolysis). Ovulation occurred in nine of nine heifers in the vehicle group and in three of 10 heifers in the FM group. The anovulatory follicles in the FM group grew to 36.2 ± 2.9 mm, and the wall became thickened from apparent luteinization. The hypothesis that PGF2α was involved in the continued P4 decrease and structural CL regression during postluteolysis was not supported. The hypotheses that pulses of PGFM and PRL were temporally related and that systemic FM treatment induced an anovulatory follicle were supported.

INTRODUÇÃO GERAL

A fertilidade de um rebanho bovino afeta a produtividade e lucratividade da atividade pecuária. Necessários aumentos na eficiência reprodutiva dependem da compreensão dos mecanismos endócrinos, celulares e moleculares envolvidos nos eventos do ciclo estral, e que resultam em maiores taxas de concepção. Na espécie bovina, a mortalidade embrionária é uma das causas mais relevantes da baixa fertilidade, promovendo altas perdas econômicas na atividade pecuária. As principais perdas embrionárias ocorrem nas duas a três semanas após a ovulação. Durante este período ocorre o reconhecimento materno da gestação, que compreende mecanismos que visam a inibição da regressão do corpo lúteo (CL). O reconhecimento materno da gestação requer ambiente uterino otimizado, o qual depende da função luteal em secretar adequadas quantidades de progesterona (P4).

A lise ou regressão do CL (luteólise) determina a redução da secreção de P4 (fim da fase luteal) e é importante para que a fêmea bovina retorne a novo ciclo reprodutivo após a não constatação da gestação pelo animal. O processo de luteólise envolve diversos mecanismos anatômicos, endócrinos e moleculares que estimulam a secreção de agentes luteolíticos, determinando o fim da funcionalidade e da estrutura física que compreendia o CL. Assim, o conhecimento do processo de luteólise dentro da fisiologia reprodutiva na vaca é importante não só para o controle farmacológico do ciclo estral, mas também para compreensão do reconhecimento materno da gestação e formulação de estratégias anti-luteolíticas que visem manter a gestação. Neste sentido, estudos de cunho fisiológico enfocando a elucidação dos mecanismos que

governam a luteólise sejam por indução, atraso ou bloqueio da luteólise, se constituem a base para o desenvolvimento de novas biotecnologias reprodutivas que incrementem o manejo reprodutivo e a fertilidade em bovinos.

Desta maneira, objetiva-se com o presente estudo avaliar os efeitos da inibição da secreção de PGF2α antes, durante e após o período luteolítico na regressão funcional e estrutural do CL em bovinos; elucidar o papel do estradiol em diferentes níveis fisiológicos na secreção de PGF2α e indução da luteólise; e estudar o efeito da inibição de prostaglandinas durante o período pós-luteolítico sobre o crescimento folicular e ovulação.

CAPÍTULO 1 - REVISÃO DE LITERATURA

1. Ciclo estral em bovinos

O ciclo estral compreende os eventos endócrinos, morfológicos e comportamentais no período entre duas ovulações sucessivas. A espécie bovina é classificada como poliéstrica devido a periodicidade constante dos ciclos estrais ao longo do ano [Youngquist & Threlfall, 2007]. Neste contexto, o ciclo estral resulta principalmente da interação do sistema nervoso central, hipotálamo-hipófise, útero e ovário. A comunicação entre esses órgãos e tecidos ocorre através da participação de vários hormônios, fatores de crescimento e seus receptores, os quais irão determinar o tempo de duração do ciclo estral. Em média, o ciclo estral em bovinos envolve três semanas de duração, mas pode variar entre 18 a 24 dias [Wishart, 1972; Sirios & Fortune, 1988; Ginther et al., 1989]. A ciclicidade reprodutiva se inicia na puberdade e geralmente continua inalterada, menos quando a gestação é estabelecida ou em condições nutricionais ou patológicas limitantes.

Na vaca, o tempo de permanência do corpo lúteo (CL) é o maior determinante da variação na duração do ciclo estral [Sirois & Fortune, 1988; Ginther et al., 1989]. Durante a permanência do CL os folículos ovarianos crescem sob a dinâmica de ondas de crescimento e não são capazes de ovularem devido ao efeito inibitório da secreção de progesterona (P4) luteal sobre a secreção gonadotrópica da hipófise [Ginther et al., 1989; Sirois & Fortune, 1990]. Assim, quanto maior a persistência do CL, maior número de ondas será verificado durante o ciclo estral. Após a regressão do CL, o folículo

selecionado na fase de divergência folicular pode desenvolver-se até a ovulação, marcando o térmico do ciclo estral e início de uma nova onda de crescimento folicular [Ginther et al., 1989]. Geralmente, duas ou três ondas de crescimento folicular são verificadas durante o ciclo estral [Ginther et al., 1989; 1996], mas ciclos estrais com uma ou quatro ondas também ocorrem em menor frequência. Esta variação pode ocorrer em função de vários fatores, como dieta, manejo, produção de leite, período de lactação e período pós-parto imediato [Ginther et al., 1996].

Neste contexto, o ciclo estral pode ser dividido em duas fases [Youngquist & Threlfall, 2007] – luteal (formação do CL até sua regressão) e folicular (da regressão luteal até a ovulação). Além disso, o ciclo estral pode ser separado em quatro estágios – estro (dia 0), metaestro (dia 1 a 3), diestro (dia 4 a 18), e proestro (dia 19 até manifestação de estro). O estro é caracterizado pelo comportamento sexual de receptividade da fêmea (comportamento de imobilização) a um touro ou por outras fêmeas, em adição ao crescimento folicular e maior secreção esteroidogênica. O metaestro é caracterizado pela maturação final do folículo e ovulação, com formação de um CL jovem e subsequente habilidade para secretar P4. Aumento siginificativo nas concentrações de P4 são observadas na fase luteal ou período de diestro que se extende até o início da regressão do CL (luteólise). As concentrações de P4 declinam rapidamente com a luteólise, e assim nova fase folicular é iniciada, promovendo a seleção e crescimento acelerado de um folículo dominante. Desta forma, a fase de proestro é marcada por gradual aumento das concentrações de estradiol (E2) promovido pelo crescimento e maior capacidade secretória do folículo dominante.

2. Importância e síntese das prostaglandinas durante o ciclo estral de fêmeas bovinas

As prostaglandinas (PGs) foram descobertas em 1930 com os trabalhos de Kurzrok & Lieb, que descreveram os efeitos que o sêmen humano exerce sobre o miométrio [Simmons et al., 2004]. Em 1935, Von Euler, foi o primeiro a identificar o princípio ativo responsável por tais ações, denominando-o de

prostaglandina, ao acreditar erroneamente, que sua síntese era realizada na próstata, e recebendo o sufixo “glandinas” por estar associado à uma glândula (revisado em [Simmons et al., 2004]). Mais tarde, pesquisadores do Instituto Karolinska de Estocolmo, separaram a primeira PG (PGF2α) na forma pura cristalina, a partir de vesículas seminais de carneiros, e em 1962 conseguiram estabelecer sua estrutura química. Assim, estabeleceram que as PGs são ácidos graxos hidroxilados não saturados de 20 carbonos, com um anel ciclopentano no C8 – C12 e são parte do grupo denominado eicosanóides. Atualmente, as PGs são classificadas em diversas classes de acordo a estrutura do anel ciclopentano. Dentro destas classes, as principais PGs associadas com os eventos do ciclo estral são a PGF2α e a PGE2.

As PGs impactam sobre as funções ovariana, uterina, placentária e hipofisária envolvidas na regulação da reprodução em fêmeas domésticas. Estas substâncias desempenham importantes papéis na ovulação, função luteal, reconhecimento materno da gestação, abortamento, parto, infecções uterinas, e retorno da ciclicidade ovariana no pós-parto [Weems et al., 2006]. As PGs possuem tanto efeitos positivos quanto negativos na reprodução, e assim, são usadas para diversas finalidades como sincronização de estro, tratamentos de pseudogestação, indução de parto, retenção de placenta, cistos luteinizados, piometra, e endometrites crônicas [Weems et al., 2006]. Desta maneira, o aprimoramento dos usos terapeuticos das PGs é obtido através do melhor conhecimento dos processos fisiológicos e reprodutivos em que elas atuam. Assim, para o entendimento da função das PGs na reprodução necessita-se compreender seus mecanismos de síntese e metabolismo.

Neste contexto, a biossíntese da PGF2α e a PGE2 envolve a liberação do ácido araquidônico a partir dos fosfolipídeos de membrana celular (principalmente fosfatidilcolina e fosfatidiletanolamina), que é catalizada pela enzima citosólica fosfolipase A2 (Figura 1 [Simmons et al., 2006; Clark et al., 1991]). As PGs têm sua síntese desencadeada por estímulos nas membranas celulares, que podem ser de natureza fisiológica, farmacológica ou patológica [Simmons, 2004]. Tais estímulos ativam receptores de membrana, acoplados a uma proteína regulatória ligada a um nucleotídeo guaninico (proteína G). A partir desta ligação, a fosfolipase A2 torna-se ativa. Faz parte deste complexo

ainda, uma elevação da concentração de cálcio (Ca++) no compartimento intracelular. O ácido araquidônico, oriundo da reação hidrolítica mediada pela fosfolipase A2, é então substrato para duas vias enzimáticas, a das cicloxigenases, que desencadeiam a síntese das PGs e tromboxanos, e a via das lipoxigenases, responsável pela síntese dos leucotrienos.

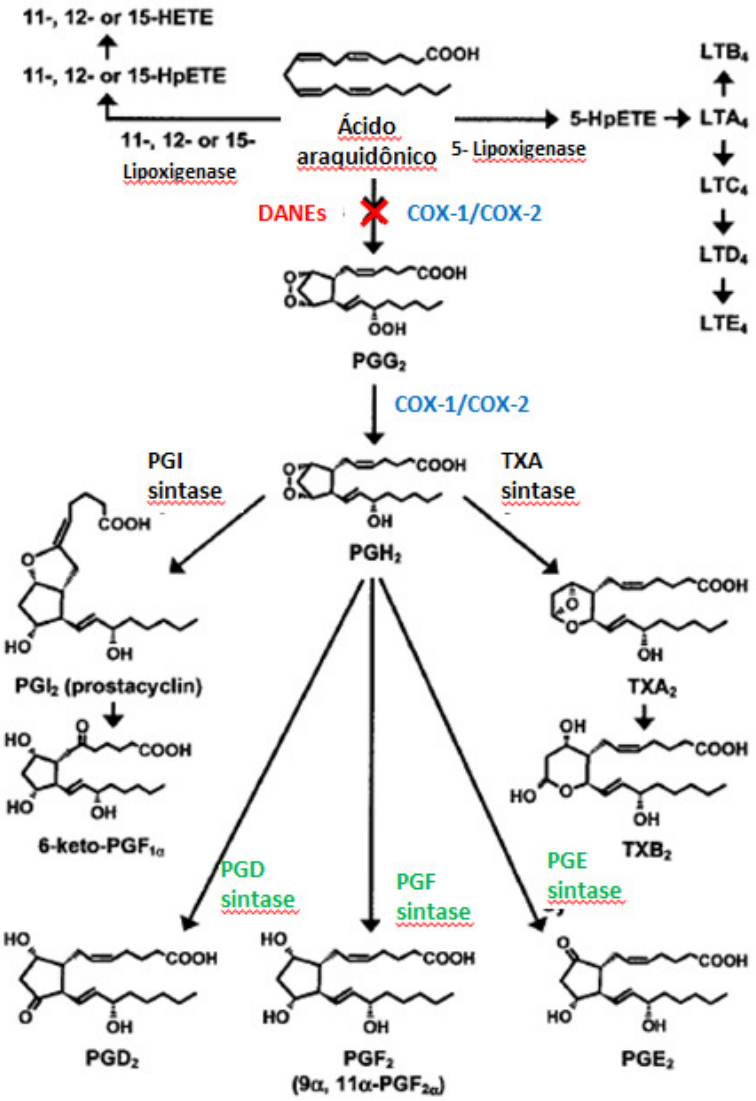

Figura 1: Esquema de formação das prostaglandinas a partir do ácido araquidônico. O ácido araquidônico é metabolizado pelas cicloxigenases (COX 1 e COX 2) em prostaglandina H2 (PGH2) via formação da prostaglandina G2 (PGG2). A PGH2 espontaneamente ou enzimaticamente é convertida em PGD, PGE, PGF, PGI e tromboxanos (TX). Drogas antinflamatórias não-esteroidais (DANEs) inibem a síntese de PGG2. Adaptado de Simmons et al., 2006 .

Para a síntese de PGs, o ácido araquidônico livre é convertido em PGH2 pela enzima cicloxigenase (COX) através de uma conversão oxidativa [Smith & Lands, 1972; Hamberg et al., 1974]. O local de atividade das COX é um canal hidrofóbico que se encontra numa estrutura entre α-hélices associadas à membrana. A síntese inicia-se com a COX catalisando a adição de oxigênio molecular ao ácido araquidônico, formando-se um produto intermediário, a PGG2. A conversão do ácido araquidônico é considerado a etapa limitante na produção de PGs. Em outro sítio ativo da molécula, a COX cumpre sua função como peroxidase, reduzindo a PGG2 a PGH2. A PGH2 é o precursor da PGF2α e a PGE2 [Simmons et al., 2004]. A conversão de PGH2 em PGF2α e PGE2 envolve ainda a partipação, respectivamente, das enzimas prostaglandina F sintase e prostaglandina E sintase.

3. Corpo lúteo e esteroidogênese luteal

O corpo lúteo (CL) é um órgão endócrino temporário cuja principal função é a secreção de P4 pelas células esteroidogênicas [Niswender et al., 2000; Skarzynski et al., 2008; Salles & Araujo, 2010]. A cada ciclo estral, as células luteínicas esteroidogênicas sintetizam e liberam P4 na circulação sistêmica, promovendo a quiescência na contratilidade do miométrio, o desenvolvimento glandular do endométrio e o ambiente uterino adequado para o desenvolvimento do concepto. A adequada função luteal em secretar P4 é crucial para determinar a duração fisiológica do ciclo estral e para ocorrer uma gestação bem sucedida.

O CL é composto por vários tipos celulares heterogêneos, que consistem não apenas de células esteroidogênicas (células luteais pequenas e grandes), mas também de células não esteroidogênicas (células endoteliais, células musculares, periócitos, fibrócitos e células imunes [Shirasuna et al., 2007]). As células endoteliais vasculares representam mais de 50% do total do número de células no CL [O'Shea et al., 1989; Lei et al., 1991] e são responsáveis pela secreção de muitas substâncias vasoativas que regulam diretamente a secreção de P4 luteal [Myamoto et al., 1993, 1997; Shirasuna et al., 2007]. Diversos fatores angiogênicos são produzidos pelo CL bovino, como fator de

crescimento vascular endothelial (VEGF), fator de crescimento fibroblático básico (bFGF) e angiopoetina 1 e 2 (ANPT-1 e 2) [Miyamoto et al., 2009]. Além disso, substâncias vasoativas como a endotelina-1 (EDN1), angiotensina II (Ang II), óxido nítrico e a propria PGF2α são produzidas no CL bovino [revisado em Miaymoto et al., 2009]. Assim, os vasos sanguíneos e as células endoteliais no CL têm um papel essencial na função luteal nas vacas.

De acordo com estudos morfológicos e imunológicos em bovinos, acredita-se que as células luteais grandes originem-se das células da granulosa e as células luteais pequenas das células da teca interna do folículo ovulatório [Niswender et al., 2000]. As células luteais pequenas apresentam diâmetro entre 12 e 22 µm, grande quantidade de mitocôndrias, retículo endoplasmático liso e gotas lipídicas no citoplasma e grande número de receptores de hormônio luteinizante (LH), sendo altamente responsivas ao estímulo desse hormônio para a secreção esteroidogênica. Estas células secretam E2 e baixas quantidades de P4, e constituem aproximadamente 20 a 30% do volume do CL, representando 25% do total das células [Bertan et al., 2006]. As células luteais grandes são esféricas, apresentando diâmetro entre 23 e 50 µm e grande quantidade de mitocôndrias, retículo endoplasmático rugoso, complexo de Golgi e grânulos secretórios no citoplasma relacionados a sua alta capacidade esteroidogênica e síntese de proteínas [Wiltbank & Niswender, 1992]. Elas são responsáveis por 80% da secreção de P4 e possuem poucos receptores para LH. Também são responsáveis pela produção de ocitocina e são sítios primários de resposta às PGs e ao E2. Elas representam 40% do volume do CL, sendo apenas 19% do total de células [Fields et al.,1992].

O CL bovino se desenvolve rapidamente entre 2 a 3 dias após a ovulação, acompanhado de grande angiogênese e vascularização [Miyamoto et al., 2009]. O desenvolvimento luteal normal e sua capacidade de produzir P4, fatores de crescimento e substâncias vasoativas dependem de sua vascularização [Acosta et al., 2004]. A maioria das células esteroidogênicas estão em íntimo contato com os capilares. Fatores de crescimento (como o VEGF e o bFGF) são os principais atuantes no desenvolvimento e na manutenção da densa rede de capilares neoformados e contribuem para a produção de P4 [Sales & Araujo, 2010].

O estímulo da esteroidogênese luteal é realizado principalmente pelo LH [Skarzynski et al., 2008]. Este hormônio luteotrófico é necessário para o desenvolvimento estrutural e funcional do CL. Outros hormônios também participam da estimulação do crescimento e da função luteal, incluindo a ocitocina, PGs e fatores de crescimento. A ocitocina tem efeito luteotrófico no início do diestro potencializando a ação do LH. A PGE2 e PGI2 tem ação luteotrófica e luteoprotetora, agindo de maneira autócrina no estímulo da produção de P4 [Niswender et al., 2000], já a PGF2α tem ação luteolítica e é fudamental no processo de luteólise [McCracken et al., 1999]. O hormônio do crescimento (GH) atua na regulação da ação des fatores de crescimento e estímulo à produção de P4 [Niswender et al., 2000, Skarzynski et al., 2008].

Após a ovulação, as células da teca e as células da granulosa, que até então sintetizavam estrógenos, são reorganizadas para formarem o CL e passam a sintetizar P4 [Shirasuna et al., 2007]. Após o pico ovulatório de LH, a produção de androstenediona e de E2 diminui e, em contrapartida, começa a incrementar-se a síntese de P4 [Alila & Dowd, 1991]. Para tal mudança de especialidade, ocorre aumento na expressão das enzimas envolvidas na conversão de colesterol em P4 (P450scc e 3ß-HSD) e decréscimo na expressão das enzimas que convertem P4 em estradiol (P45017α-hidroxilase e P450 aromatase). Neste contexto, tanto o transporte do colesterol na forma de lipoproteínas de alta (HDL) ou baixa (LDL) densidade na corrente sanguínea, como o transporte para a membrana mitocondrial das células luteais pela proteína de regulação aguda da esteroidogênese (StAR- “Steroidogenic Acute Regularoty Protein”) são fundamentais para a secreção de P4 [Niswender et al., 2000; Bertan et al., 2006].

4. Luteólise espontânea em bovinos

Na ausência da fertilização ou na incapacidade do concepto em sinalizar sua existência no útero, o CL deve regredir para possibilitar a ocorrência de novo estro [Milvae, 2000; Webb et al., 2002]. Durante o processo de lise ou regressão do CL (luteólise) o tecido luteal sofre mudanças bruscas na capacidade esteroidogênica, vascularização e remodelamento, resultando em

substituição da glândula por tecido conjuntivo [Stocco et al., 2007]. Assim, a luteólise espontânea pode ser dividida em duas fases [Stocco et al., 2007; Skarzynski et al., 2008]. A primeira é a luteólise funcional, a qual é associada à marcado decréscimo na secreção de P4. A segunda fase, conhecida como luteólise estrutural, ocorre após o início do declínio da P4 e é determinada pela apoptose celular do CL até formação do corpo albicans.

4.1. Luteólise funcional

A luteólise funcional é caracterizada pela drástica redução da secreção de P4 em curto período de tempo na ovelha [McCracken et al., 1999] e na vaca [Ginther et al., 2010a]. A luteólise funcional se inicia antes de se observar mudanças morfológicas na integridade das células luteais [Stocco et al., 2007]. Assim, considerando-se as concentrações plasmáticas de P4, pode-se classificar três períodos relacionados com a luteólise espontânea em bovinos [Ginther et al., 2010a; 2010b], o período antes do início da redução da secreção de P4 (pré-luteólise), o período de grande redução da P4 (período luteolítico) e o período após o decréscimo das concentrações de P4 a <1ng/mL (período pós-luteolítico). Baseado em amostragem da P4 plasmática de hora em hora, estima-se que a duração do período luteolítico é de 24 horas em novilhas [Ginther et al., 2010a].

A redução das concentrações de P4 durante a luteólise funcional em bovinos não ocorre de maneira contínua como observado na espécie equina [Ginther et al., 2011a; 2011b]. Em novilhas, aumentos ou flutuações nas concentrações plasmáticas de P4 ocorrem ao longo da período luteolítico [Ginther et al., 2010a; 2011b; 2011c]. Há evidências em ratos [revisado em Stocco et al., 2007] indicando que durante a regressão funcional, as células luteais estão ainda com atividade esteroidogênica, entretanto o principal esteróide secretado durante este período não se constitui a P4, mas sim um metabólito da P4 (20α-progesterona). Em bovinos, outra característica que também pode ser observada durante a luteólise funcional é a perda de receptores de LH aliado a menor fluxo sanguíneo no CL [Niswender et al., 1976]. Esta redução na irrigação luteal parece ocorrer após aumento agudo e

transitório no fluxo sanguíneo logo após a indução da luteólise com PGF2α exógena [Acosta et al., 2002]. Da mesma forma, durante um pulso luteolítico espontâneo de PGFM, o fluxo sanguíneo luteal aumenta gradativamente durante a parte ascendente do pulso até o seu pico, e mantém elevado por 2 horas durante a parte descendente do pulso, para posteriormente reduzir [Ginther et al., 2007]. Da mesma forma, durante pulsos de PGFM simulados por infusão intrauterina com PGF2α, o fluxo sanguíneo no CL aumenta dentro de 1 hora após o início da infusão em novilhas [Shrestha et al., 2010b; 2011].

4.2. Luteólise estrutural

A regressão estrutural do CL é caracterizada pelo decréscimo no tamanho e peso da glândula, a qual eventualmente se torna um tecido totalmente regredido dentro do estroma ovariano denominada corpo albicans. A involução do CL é na verdade não somente a apoptose do tecido luteal, mas também a reposição do suporte vascular e tecido conectivo com feixes de fibras colágenas, fibroblastos e macrófagos. A maioria dos corpos albicans são substituídos por estroma ovariano [Stocco et al., 2007]. Na maior parte das espécies, a distinção entre a luteólise funcional e estrutural não está claramente reportada e os dois eventos podem não ser inteiramente separados [McCracken et al., 1999]. Por exemplo, em ratos a PGF2α não causa a luteólise estrutural, a qual é induzida pela prolactina (PRL) apenas após finalizada a luteólise funcional [Behrman et al., 1993]. Em bovinos, a luteólise estrutural se inicia logo após o início do declínio da P4 plasmática [Ginther et al., 2007]. Entretanto, o processo final de apoptose e substituição do tecido luteal pode demorar de alguns dias até semanas para ser completado após o fim da luteolíse funcional [McCraken et al., 1999].

Uma grande variedade de agentes tem sido proposta como mediadores da luteólise estrutural [McCracken et al., 1999]. Macrófagos e outras células imunes possuem funções importantes na regressão luteal via a liberação do fator de necrose tumoral-α (TNF-α) e outras citocinas [Benyo & Pate, 1992]. O TNF-α pode também estar envolvido no efeito antiesteroidogênico durante a

luteólise, já que *in vitro* o TNF-α inibe a secreção de P4 estimulada pelo LH nas células luteais bovinas [Benyo & Pate, 1992].

Durante a luteólise estrutural, um remodelamento considerável do tecido luteal ocorre durante a transformação do CL para corpo albicans. As metaloproteinases tem sido sugeridas como as principais mediadoras do remodelamento tecidual, particularmente da matriz extracelular [Birkedal-Hansen, 1995]. O CL produz inibidores específicos das metalopreoteinases que auxiliam na manutenção da integridade estrutural da glândula. A mais abundante metaloproteinase no CL de bovinos [Smith et al., 1996] é a metaloproteinase-1 (TIMP-1). Na vaca, a expressão luteal da TIMP-1 aumenta em 24 horas após a indução da luteólise com PGF2α exógena [Smith et al., 1996]. O efeito positivo da PGF2α sobre a ação de algumas caspases e em citocinas correlacionadas com a defragmentação de DNA celular também tem sido associado aos mecanismos que controlam a sinalização apoptótica durante a luteólise estrutural [revisado em Stocco et al., 2007].

4.3. Mecanismos anatômicos, endócrinos e moleculares envolvidos na luteolíse em bovinos

O entendimento dos mecanismos luteolíticos requer o conhecimento das relações anatômicas entre o ovário, útero e suas vascularizações associadas. A exposição do útero à interação coordenada entre P4, E2 e ocitocina [Hooper et al., 1986; Lefrance & Goff, 1988; Silvia et al., 1991], aliada à expressão de seus receptores no útero [Mann et al., 2001; Mann & Lamming, 2006], são essenciais para a secreção pulsátil de PGF2α pelo endométrio, a qual induz a luteólise [McCracken, 1999]. Além do E2, P4 e ocitocina, outros hormônios (LH, PRL, cortisol) e várias substâncias vasoativas (óxido nítrico, VEGF, EG-VEGF, bFGF, END-1, AngII) estão envolvidas no processo de regressão do CL [Stocco et al., 2007].

Neste contexto, verifica-se que o útero apresenta papel fudamental no controle da luteólise. A importância uterina foi primeiramente reportada em roedores [Loeb, 1923; 1927], através da inexistência de ciclos reprodutivos e a persistência do CL após a histerectomia. Efeitos similares também foram

observados em ovinos, bovinos, suínos e equinos [Anderson et al., 1969]. Já em primatas, este efeito não é observado [Beavis et al., 1969; Neill et al., 1969]. A persistência luteal após a histerectomia nas espécies supracitadas indicaram que o útero é o responsável pela produção de uma substância que causa a regressão cíclica do CL. Todas as espécies que mantiveram o CL após esse procedimento possuem um útero bicornuado [McCracken et al., 1999]. Na maioria destes animais, o efeito unilateral da histerectomia foi observado, ou seja, manutenção do CL após retirada de apenas um corno uterino. Posteriormente, evidências indicaram que a PGF2α é esta substância luteolítica, que é liberada ciclicamente pelo útero por volta dos dias 15, 16 ou 17 após a ovulação [Thatcher et al., 2001], e é o principal determinante na indução espontânea da luteólise em bovinos [McCracken, 1999]. A concentração de PGF2α pode ser representada pela mensuração da concentração de seu metabólito 13,14-dihidro-15-ceto- PGF2α (PGFM [Ginther et al., 2007]).

Um mecanismo local de transferência da PGF2α existe no pedículo útero-ovariano da vaca [Ginther et al., 1966; 1967; Hixon & Hansen, 1974]. Estudos envolvendo a ligação dos vasos sanguíneos do útero e do ovário indicaram a importância da via vascular no efeito local do útero sobre a função ovariana em diversas espécies (revisado em [McCracken et al., 1999]). A artéria ovariana é fortemente anexada a superfície da veia útero-ovariana e percorre a veia de forma tortuosa antes de sua entrada no hilo ovariano. Esta via local facilita que substâncias difundam da veia útero-ovariana para a artéria ovariana e alcançem o ovário diretamente sem passar pela circulação sistêmica [Ginther, 1981]. Além desse mecanismo de ação local, há evidências de que a PGF2α possa agir, em parte, pela via sistêmica na vaca, já que ciclos estrais foram observados em bovinos de corte após a hemi-histerectomia quando o lado que o CL estava presente não foi considerado [Ward et al., 1976]. Adicionalmente, ciclos estrais de duração normal foram observados quando o ovário foi transplantado através de anastomoses vasculares com a artéria carótida e a veia jugular na vaca, e o outro ovário removido [Barcikowski et al., 1976].

Outra evidência que demonstra a maior eficácia da via local na indução da luteólise está relacionada à dose de PGF2α necessária para induzir a

regressão do CL. A dose mínima de PGF2α (1-2 mg) quando administrada via intrauterina (IU) no corno uterino ipsilateral ao CL é em torno de 10 vezes menor do que a dose via sistêmica (15 mg), a qual é administrada via endovenosa [Louis et al., 1974; Lauderdale & Fokolowsky, 1979]. Neste contexto, a injeção de PGF2α (6 mg) dentro do corno uterino é seguido por maiores concentrações de PGF2α na artéria ovariana ipsilateral do que na artéria carótida [Hixon & Hansel, 1974]. Assim, a rota natural de transporte entre o útero e ovário deve ser considerada em estudos da luteólise que utilizem PGF2α exógena. Além disso, há a complexidade de se utilizar a veia jugular para tratamento com PGF2α ou para amostragem sanguínea já que 65% da PGF2α é metabolizada durante uma única passagem pelos pulmões na vaca [Davis et al., 1985].

4.3.1. Ação da prostaglandina F2α

A secreção de PGF2α pelo útero determina o final da fase luteal em várias espécies domésticas [McCracken et al., 1999; Weems et al., 2006; Skarzynski et al., 2008]. Em bovinos, a secreção de PGF2α ocorre em vários pulsos (4-7 pulsos) de curta duração (3-6 horas) durante 2 a 3 dias, ocorrendo previamente, durante e após o declínio da P4 circulante [Kindhal et al., 1981; Ginther et al., 2007; 2010b]. Pulsos sequenciais de PGF2α são requeridos para induzir a luteólise completa em bovinos [Ginther et al., 2009a], equinos [Ginther et al., 2009b] e ovinos [Schramm et al., 1983]. A necessidade do estímulo sequencial da PGF2α para promover a luteólise foi demonstrada em bovinos [Ginther et al., 2009a] através da ocorrência de luteólise completa (P4 < 1ng/mL) após três sessões de infusão de PGF2α (0,5 mg via IU a cada 12 horas), sendo que as concentrações de P4 resurgem quando realizado apenas uma sessão.

Devido aos vários efeitos adversos e respostas não fisiológicas após a administração de doses farmacológicas de PGF2α para induzir a luteólise [Ginther et al., 2009a; 2009b], o estudo das características da luteólise espontânea em bovinos deve ser realizado utilizando-se doses fisiológicas de PGF2α e seus análogos, e em tempo e intervalos que se assemelham aos

pulsos espontâneos. Um exemplo destes efeitos é que a administração de única dose luteolítica de PGF2α promove início mais rápido e reduz a duração normal da luteólise funcional em ovinos [McCracken et al., 1984] e bovinos [Ginther et al., 2009a]. Assim, a administração de doses fisiológicas de PGF2α em intervalos próximos aos intervalos dos pulsos espontâneos de PGF2α se torna uma técnica mais interessante no estudo do efeito da PGF2α sobre a luteólise. Neste contexto, a dose total de PGF2α requerida para causar a luteólise funcional, quando administrada de maneira pulsátil, é de 1/10 a 1/40 da dose mínima requerida quando administrado de maneira contínua [Schramm et al., 1983; Ginther et al., 2007].

A pulsatilidade da secreção de PGF2α durante os períodos pré-luteolítico, luteolítico e pós-luteolítico pode ser mensurável pelo aumento na concentração de PGFM [Ginther et al., 2007]. Durante o período luteolítico, os pulsos espontâneos de PGFM são mais proeminentes (pico e amplitude dos pulsos são maiores) do que os pulsos durante o períodos pré- e pós-luteolítico [Ginther et al., 2010a; 2010b]. Durante a parte ascendente do último pulso de PGFM no período pré-luteolítico, a concentração de P4 decresce para o mais baixo nível no pico do pulso de PGFM, seguido por aumento de P4 (efeito rebote) para as concentrações prévias ao pulso [Ginther et al., 2010c; 2011c]. O efeito rebote completo durante o pulso de PGFM no período pré-luteolítico é semelhante ao rebote em P4 que ocorre durante o pulso de PGFM induzido pelo E2 [Ginther et al., 2010d; Imam et al., 2010], e também é similar ao rebote completo que ocorre uma ou duas horas após o início da infusão de PGF2α para simular o pulso de PGFM [Shrestha et al., 2010a; 2010b; 2011]. Durante o início do período luteolítico, a P4 também decresce durante a parte ascendente do pulso de PGFM, entretanto, a concentração de P4 durante o rebote não retorna para as concentrações prévias [Ginther et al., 2010a; 2010c]. No período luteolítico avançado o efeito rebote não é mais observado. O pico do último pulso de PGFM durante a pré-luteólise ocorre em média 4 horas (variação de 2 a 8 horas) antes da hora de transição entre pré-luteólise e luteólise e o primeiro pulso durante a luteólise ocorre 9 horas após a transição [Ginther et al., 2011c]. Desta maneira, estes estudos indicam que o último pulso de PGF2α durante a pré-luteólise reduz temporariamente a secreção de P4 e inicia o subsequente início da luteólise.

O sinal luteolítico promovido pela secreção inicial de PGF2α promove a cascata luteolítica envolvendo uma série de reguladores da função luteal, como o fluxo sanguíneo local, citocinas e fatores de crescimento. O efeito inicial da PGF2α na esteroidogênese luteal é mediado pelo receptor de membrana ligado a proteina G [Davis & Rueda, 2002]. A ativação desse receptor pela PGF2α promove a produção de inusitol trifosfato (IP3) e diaciglicerol (DAG) a partir do fosfatidilinositol-4,5-difosfato mediado pela ação da fosfolipase C [Davis et al., 1987]. O aumento do IP3 e do DAG é seguido pelo aumento do cálcio intracelular livre e atividade da proteína quinase C [Davis et al., 1987]. Adicionalmente a ativação da fosfolipase C, há evidências de que a PGF2α ativa a via da fosfolipase D, produzindo ácido fosfatídico em adição ao DAG, e ativa a cascata sinalizadora da MAP quinase [Tai et al., 2001; Chen et al., 2001].

O decréscimo rápido do fluxo sanguíneo luteal tem sido proposto como uma das principais ações da PGF2α. Mudanças rápidas nos fatores angiogênicos diretamente envolvidos com o início da regressão vascular e modulação da secreção de P4 são causadas pela PGF2α [Miyamoto et al., 2009]. Inicialmente, o fluxo sanguíneo luteal na área periférica do CL aparentemente é aumentada rapidamente como resultado da vasodilatação causada pelo óxido nítrico [Acosta et al., 2002; Miyamoto et al., 2009]. Isto sugere que o aumento do fluxo sanguíneo luteal causado pela PGF2α é um dos primeiros sinais do início da cascata luteolítica na vaca. Na fase inicial da luteólise, a PGF2α suprime drasticamente a expressão de VEGF no CL. Outras substâncias vasoativas, como a EDN1, Ang II e a PGF2α de origem luteal aumentam no CL, aonde severa vasoconstricção é induzida para interromper o suporte sanguíneo e acelerar a cascata luteolítica (revisado em [Miyamoto et al., 2009]).

O mecanismo pelo qual a PGF2α reduz os níveis de P4 secretado pelo CL ainda precisa ser mais bem elucidado em bovinos. Em ratos, tem sido demonstrado [Strauss & Stambaugh, 1974; Kawano et al., 1988] que a PGF2α não inibe a síntese de P4, mas, ao invés, causa o seu metabolismo para 20α-de-hidro-progesterona. A administração de PGF2α exógena em bovinos causa a redução de RNAm para fatores relacionados a síntese esteroidogênica (StAR, 3β-hidroxiesteróide dehidrogenase - 3β-HSD) e fatores agiogênicos

(VEGF, Ang1, IGF-I e IGF-II) e aumenta o RNAm para fatores relacionados a síntese de PGs (COX-2 e enzima prostaglandina sintase [Shirasuna et al., 2010]). O efeito positivo da PGF2α sobre a ação de algumas caspases e em citocinas correlacionadas com a defragmentação de DNA celular tem sido associado aos mecanismos que controlam a sinalização apoptótica durante a luteólise estrutural (revisado em [Stocco et al., 2007]). Desta maneira, a ação da PGF2α na luteólise é mediada por fatores locais que atuam na síntese de P4 e na regressão estrutural das células luteais [Skarkzynski et al., 2008].

4.3.2. Papel dos hormônios esteroidais e da ocitocina

O E2, P4 e a ocitocina têm sido correlacionados como reguladores do início da secreção de PGF2α durante a luteólise em fêmeas bovinas. Atualmente, vários modelos de mecanismos controladores da luteólise têm sugerido mudanças na expressão endometrial de receptores de E2, P4 e ocitocina influenciando na regulação da secreção de PGF2α uterina e consequente luteólise na vaca [Silvia et al., 1991; Arosh et al., 2004; Weems et al., 2006]. Nestes modelos, a expressão de receptores de E2 e de ocitocina é suprimida nas fases inicial e intermediária de desenvolvimento do CL devido ao efeito inibitório da alta concentração de P4 [Wathes & Hamon, 1993; Ivell et al., 2000]. O efeito da P4 sobre a sensibilidade uterina à ocitocina envolve uma ação não genômica da P4 no receptor uterino de ocitocina. A P4 se liga ao receptor de ocitocina com alta afinidade, inabilitando a molécula de ocitocina ter capacidade de se ligar, e consequentemente suprimindo a indução da produção de IP3 e mobilização de cálcio necessários para a resposta celular [Zingg et al., 1998]. A exposição do útero à P4 por no mínimo 10 dias após a ovulação é necessário para ativar os mecanismos de secreção espontânea de PGF2α [McCracken et al., 1984; Silvia & Raw, 1993]. A prolongada exposição a P4 promove o acúmulo de ácido araquidônico e COXs nas células endometriais, necessárias para síntese de PGF2α.

Durante o final da fase luteal, os receptores de E2 no útero aumentam após o decréscimo dos receptores de P4 e decréscimo da sensibilidade uterina à P4

[Meyer et al., 1988]. Sequencialmente, a ação do E2 circulante sobre os seus receptores endometriais estimula a síntese de receptores de ocitocina com subsequente secreção de PGF2α através da ação da ocitocina em seus receptores endometriais [Flint & Sheldrick, 1985; Silvia et al., 1991; Mann et al., 2001]. A fonte de E2 durante a luteólise é oriundo dos folículos ovarianos, especialmente os de maior diâmetro e/ou futuros folículos dominantes que irão se desenvolver para folículos ovulatórios [Beg & Ginther, 2006]. Na vaca, o aumento inicial de receptores de ocitocina que precede a luteólise ocorre após o 13º dia pós-ovulação [Mann & Lammming, 1994].

O aumento da proeminência (pico e amplitude) dos pulsos de PGF2α após a transição entre a pré-luteólise e luteólise [Ginther et al., 2010a; 2010b] é temporariamente associado com o aumento na concentração de E2 circulante [Ginther et al., 2010c; 2011c]. A secreção de PGF2α, representada pelas concentrações de PGFM, é induzida dentro de 6 a 8 horas após o tratamento com E2 exógeno em novilhas [Thatcher et al., 1986; Araujo et al., 2009]. Um único tratamento com 0,1 mg de E2 no dia 14 pós-ovulação [Ginther et al., 2010d; Iman et al., 2010] estimula um pulso proeminente de PGFM e a luteólise prematura.

Em ruminantes, pulsos de ocitocina (ou da sua neurofisina associada) ocorrem concomitantes com pulsos de PGF2α durante a luteólise [Fairclough et al., 1980; Webb et al., 1981]. A maioria destes pulsos de ocitocina aparentemente são secretados pelo CL [Walters et al., 1984; Hooper et al., 1986; Moore et al., 1986]. A PGF2α secretada pelo útero e a ocitocina luteal compõem um sistema de retroalimentação positiva. Em bovinos, a administração de PGF2α estimula a secreção de ocitocina no CL [Flint & Sheldrick, 1982; Lamsa et al., 1989], assim como altas doses de ocitocina promovem o aumento da secreção de PGF2α [Silvia & Taylor, 1989; Kotwica et al., 1998]. Entretanto, os fatores que iniciam e terminam a secreção destes hormônios ainda não foram determinados.

Durante os pulsos espontâneos de PGF2α, a concentração de PGF2α no efluente venoso útero-ovariano aumenta antes de qualquer aumento de ocitocina seja detectado [Moore et al., 1986]. Isto implica que apesar de não se saber o que promove o início da secreção de PGF2α, a ativação do sistema de

retroalimentação positiva inicia-se no lado uterino. Duas possibilidades tem sido estudadas como causa do estímulo da secreção inicial de PGF2α. A primeira considera que a o útero possui um centro rítmico endógeno que promove a secreção de PGF2α a cada 6-8 horas de intervalo. A outra possibilidade é que o útero recebe sinal pulsátil externo oriundo de outro tecido. O principal candidato em prover essa sinalização é a ocitocina secretada pela hipófise posterior [Silvia et al., 1991]. Apesar da sincronização dos pulsos de PGF2α e ocitocina, a precisa relação temporal da ocitocina secretada pela hipófise posterior e da PGF2α uterina ainda não foi caracterizada.

Com base nestes aspectos relacionados anteriormente, foi proposto por McCracken et al. (1995) um modelo hipotético que explica como ocorreria o controle neuroendócrino da secreção pulsátil de PGF2α durante a luteólise. Modelo: 1) No período luteal tardio, a perda da ação da P4 ocorre devido a redução da expressão de receptores de P4 tanto no hipotálamo quanto no endométrio, o que resulta no retorno da ação do E2 sobre estes tecidos; 2) retorno da ação estrogênica irá estimular o centro hipotalâmico gerador dos pulsos de ocitocina a secretar a ocitocina em pulsos de alta frequência e baixa concentração, e simultaneamente estimular a expressão de receptores de ocitocina no útero; 3) baixos níveis de PGF2α (subluteolítico) serão secretados pelo útero devido a interação da ocitocina de origem hipofisária com os seus receptores endometriais; 4) baixas concentrações de PGF2α são suficientes para iniciar a liberação de ocitocina luteal através da ação sobre os receptores de alta sensibilidade; 5) secreção de ocitocina suplementar irá amplificar a secreção de PGF2α endometrial; 6) secreção de PGF2α pelo endométrio será, neste momento, alta o suficiente para ativar os receptores de PGF2α de baixa sensibilidade e irá inibir a secreção de P4 e liberar mais ocitocina do CL. Tais hipóteses ainda precisam ser melhor estudadas em fêmeas bovinas.

4.3.3. Ação do hormônio luteinizante

O efeito negativo da secreção de PGF2α sobre a concentração de P4 é balanceado contra um efeito positivo das oscilações de LH durante o período

final da preluteólise e período inicial da luteólise [Ginther et al., 2010c; 2011c]. O balanço periódico entre o efeito luteolítico da PGF2α e o luteotrófico do LH é compatível com os seguintes achados em novilhas: 1) o LH exógeno prolonga a vida luteal [Donaldson & Hansel, 1965]; 2) o LH é essencial para secreção de P4 pelo CL [Quintal-Franco et al., 1999]; 3) aumento das concentrações de LH no momento do rebote de P4 durante pulsos de PGFM induzidos pelo E2 [Imam et al., 2010]; e 4) o LH aumenta após a infusão de PGF2α [Shrestha et al., 2010b; 2011]. Durante a fase intermediária do desenvolvimento luteal, um pulso de LH com pico de baixa frequência e alta amplitude ocorre a cada 4 horas [Cupp et al., 1995], e é associado com aumento na P4 [Procknor et al., 1986]. A ritimicidade entre as oscilações de P4 e LH durante a preluteólise é perdida e a proeminência das flutuações de P4 são reduzidas quando as oscilações de LH são bloqueadas por um antagonista de GnRH [Ginther et al., 2011b].

O estímulo do LH na secreção de P4 não se torna eficiente no transcorrer da transição entre pré-luteólise e luteólise. Isto é indicado pela redução na ocorrência do pico de LH no mesmo momento que o pico da flutuação de P4 na luteólise (29%) comparado a preluteólise (77%) e pela não observação do efeito rebote completo nas concentrações de P4 após o pico de PGFM e associado temporalmente ao aumento de LH no período luteolítico [Ginther et al., 2010e]. A redução na sincronia entre os picos dos pulsos de LH e das flutuações de P4 durante a luteólise pode refletir a menor responsividade ao LH das células luteais em regressão.

A interação do LH em relação as concentrações do E2, ocitocina e PGF2α, e seu efeito na secreção de P4 estão ilustrados na Figura 2.

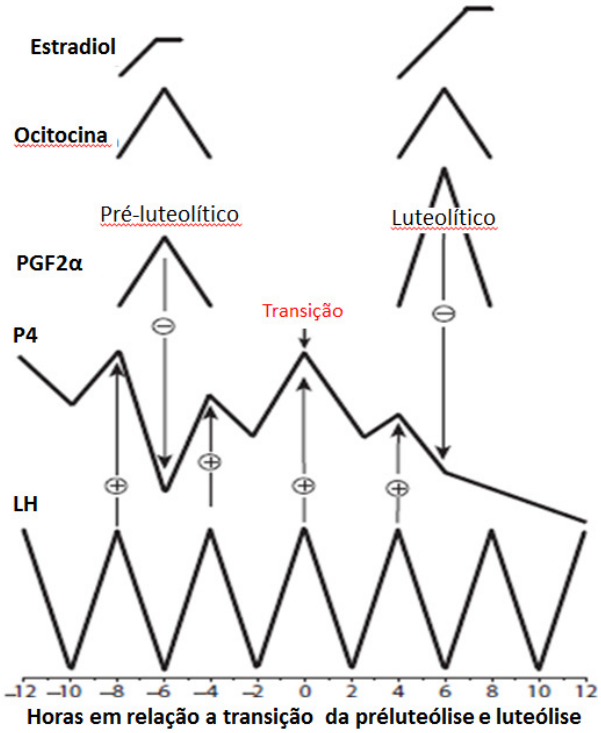

Figura 2. Modelo esquemático das interações das concentrações de estradiol e dos pulsos de ocitocina, PGF2α e LH com a secreção de P4 durante o período pré-luteolítico e luteolítico em bovinos (Cedido por OJ Ginther).

4.3.4. Função da prolactina

Além dos hormônios supracitados, outros hormônios como a prolactina (PRL) podem exercer funções específicas no mecanismo luteolítico. Em ratos, após o fim da secreção de P4, a PRL induz a apoptose das células luteais para se completar a regressão estrutural [Rothchild, 1981]. Ao contrário, tem sido reportado que a infusão *in vitro* de PRL em ovários bovinos aumenta a secreção de P4 [Bartosik et al., 1967], e em carnívoros, a PRL é sugerida como o mais importante constituinte no complexo luteotrófico [Murphy et al., 1985].

Locais de ligação para PRL foram reportados [Poindexter et al., 1979] no CL bovino, sugerindo que a PRL possua função durante a fase luteal do ciclo estral. A expressão de PRL e de RNAm para seus receptores varia durante a fase luteal [Shibaya et al., 2006] e pode estar relacionada com a regulação da secreção de P4 [Thompson et al., 2011]. Adicionalmente, segundo indicado por resultados *in vitro* [Erdman et al., 2007], a PRL pode atuar como um fator vasoativo, mediando o desenvolvimento e regressão luteal.

A ocorrência de pulsos proeminentes de PRL foi recentemente reportada durante e após a luteólise funcional em novilhas [Ginther and Beg, 2011] e éguas [Ginther et al., 2011d]. Em bovinos, esta secreção pulsátil de PRL é mais proeminente e rítmica durante as 12 horas antes do fim da luteólise e durante as primeiras 12 horas da pós-luteólise [Ginther and Beg, 2011]. A duração de um pulso de PRL é por volta de 4 horas [Ginther and Beg, 2011]. Os pulsos de PRL após o início da luteólise são temporariamente associados com o aumento das concentrações de PGFM em novilhas [Ginther and Beg, 2011] e éguas [Ginther et al., 2011d]. Centralizado ao pico de PGFM, a concentração de PRL é maior no pico e após uma hora deste, indicando aparentemente efeito positivo da PGF2α na secreção de PRL em bovinos [Ginther and Beg, 2011]. Entretanto, ainda não é conhecido se um hormônio estimula o outro ou se outros fatores estão envolvidos nesta relação temporal.

5. Inibição da secreção de prostaglandina F2α e da luteólise por drogas anti-inflamatórias não-esteroidais

Drogas anti-inflamatórias não-esteroidais (DANEs) têm sido usadas como potentes medicamentos analgésicos/anti-inflamatórios/anti-piréticos desde 1989, quando a aspirina foi colocada no mercado [Simmons et al., 2004]. Posteriormente em 1999, drogas mais seletivas à COX-2 foram desenvolvidas e também disponibilizadas para comercialização. Todas DANEs são potentes inibidores da COX através da competição com o ácido araquidônico pelos sítios ativos de ligação na molécula COX (Figura 1). Atualmente, diversas DANEs com diferentes especificidade e potencialidade têm sido comercializadas para inúmeras finalidades terapêuticas em humanos e em animais domésticos. Diversos DANEs como a ácido acetilsalicílico, endometacina, ibuprofeno, meloxicam e nimesulida possuem efeito significativo sobre a inibição da secreção de PGF2α em bovinos [Simmons et al., 2004]. O Flunexine meglumine (FM), é um dos mais comuns DANEs usados em animais de produção [Odensvik et al., 1998]. O FM atua tanto na COX 1 quanto na COX 2

[Tomlinson et al., 2004] para bloqueiar a síntese de PGs [Odensvik et al., 1998].

Em bovinos, após única injeção de FM (2,2 mg/kg peso vivo), as concentrações do metabólito da PGF2α (PGFM) decrescem dentro de 30 minutos e o efeito continua por aproximadamente 6 horas [Aiumlamai et al., 1990]. Adicionalmente, as concentrações de PGFM são suprimidas após o tratamento com FM (1g) duas vezes ao dia, por 5 dias no período pós-parto [Guilbault et al., 1987]. Tratamentos intensivos de FM por 7 a 10 dias, incluindo o período luteolítico em bovinos [Odensvik et al., 1998; Aiumlamai et al., 1990; Odensvik & Gustafsson, 1994], e quando administrado duas vezes ao dia em em ovelhas [Aké-Lopez et al., 2005], prolongam o tempo de vida do CL e aumentam a duração do ciclo estral.

Devido esta eficácia em reduzir a secreção de PGF2α, o uso do FM como outros DANEs foi reportado [Binelli et al., 2001; 2008] com uma das diversas estratégias antiluteolíticas objetivando obter melhores taxas de gestação em bovinos de leite e corte. A administração de FM no período crítico de reconhecimento materno da gestação (dias 15 a 17 pós-ovulação) aumenta a sobrevivência embrionária e a taxa de concepção [Guzeloglu et al., 2005; 2007]. Da mesma maneira, a administração de outras DANEs como o lisionato de ibuprofeno [Elli et al., 2001] ou o ácido acetilsalicílico [Pugh et al., 2004], previamente a transferência de embriões, aumentou as taxas de gestação em torno de 20 % em relação aos animais não tratados. Apesar de nestes últimos estudos os tratamentos não terem sido administrados no período normal de luteólise, é possível que a secreção de PGF2α resultante das manipulações do òrgãos genitais durante a inovulação de embriões estimule precocemente o mecanismo luteolítico. Assim, espera-se que a inibição da secreção PGF2α pelo tratamento prolongado com DANEs aumente a sobre-vida do CL e, assim, permita maior tempo para o desenvolvimento embrionário e reconhecimento materno da gestação. Ao contrário destes estudos, a administração do meloxicam, uma DANE mais seletiva a COX-2 e que apresenta maior meia-vida do que o FM, no dia 15 pós-ovulação, obteve efeito contrário, reduzindo a taxa de concepção comparado ao grupo não tratado (52% vs 24 % [Erdem & Guzeloglu, 2010]).

6. Utilização da ultrassonografia para avaliação da funcionalidade luteal

A avaliação precisa e consistente da condição funcional do CL é essencial para os estudos envolvendo a ação da PGF2α e dos demais mecanimos luteolíticos associados com a luteólise. A análise da concentração plasmática ou sérica da P4 é o padrão mais utilizado e representativo da função luteal [Kastelic et al., 1990; Ribadu et al., 1994; Tom et al., 1998; Battocchio et al., 1999]. Contudo, além do custo e do grande intervalo desde a colheita da amostra sanguínea até o resultado final, que impede a mensuração imediata após o exame ginecológico, esta análise não possibilita a avaliação das modificações morfológicas no CL. Baseado na alta correlação (r = 0,85) entre o tamanho do CL e a concentração de P4 [Ribadu et al., 1994; Veronesi et al., 2002], a condição funcional do CL pode ser avaliada através da palpação transretal em condições de campo. Entretanto, mesmo profissionais experientes podem erroneamente diagnosticar a presença ou ausência do CL, e, até mesmo na fase de diestro, a sensibilidade de palpação para identificar o CL não é maior que 85% [Ribadu et al., 1994]. A detecção da luteólise é ainda mais difícil de ser avaliada pela palpação, como indicado pelo valor de predição de apenas 64% [Veronesi et al., 2002].

Neste cenário, a ultrassonografia se torna ferramenta muito útil na avaliação ginecológica em bovinos por propiciar diagnóstico do CL mais preciso e confiável [Pierson & Ginther, 1984; 1987; Hanzen et al., 2000]. Durante a luteólise espontânea e induzida, o tamanho e o fluxo sanguíneo do CL decrescem durante a redução das concentrações plasmáticas de P4 em bovinos [Ginther et al., 2007; 2012; Araujo et al., 2009] e equinos [Ginther et al., 2008]. O uso de aparelhos de ultrassom que geram imagens bi-dimensionais em tempo real (B-mode) possibilitou avaliação precisa do tamanho do CL [Kastelic et al., 1990; Ribadu et al., 1994; Borges et al., 2003]. Todavia, a área ou volume mensurados pelas imagens ultrassonográficas pode não ser os parâmetros mais adequados para acessar a função luteal durante a luteólise, pois o tamanho do CL decresce mais lentamente do que as concentrações de P4 [Kastelic et al., 1990; Ribadu et al., 1994; Ginther et al., 2012]. Maior avanço foi adquirido pelo uso da ultrassonografia transretal com Doppler colorido, pois

possibilita mensurar o fluxo sanguíneo luteal que é o componente chave na regulação da função luteal [Acosta et al., 2002; Ginther, 2007].

Da mesma maneira, a ecotextura de determinado tecido reflete diretamente a sua estrutura histológica e a análise da ecogenicidade de uma imagem ultrassonográfica do tecido luteal permite estimativa do fluxo sanguíneo e da secreção de P4 no CL [Tom et al., 1998]. Diversos estudos [Herzog et al., 2008; Siqueira et al., 2009] têm utilizado tanto o tamanho e ecogenicidade do CL pelo ultrassom "B-mode", quanto a quantidade e intensidade de pixels coloridos na ultrassonografia com Doppler para acessar a funcionalidade do CL. Dentro destes parâmetros, tem sido reportado [Herzog et al., 2010] que o fluxo sanguíneo luteal, estimado pela ultrassonografia com Doppler colorido, é o indicador mais apropriado pois está altamente correlacionado com as concentrações de P4 ao longo do ciclo estral [Bollwein et al., 2002; Ginther et al., 2007b]. Esta estimativa do fluxo sanguíneo pode ser realizado por avaliação subjetiva na hora do escaneamento ou através da avaliação de imagens gravadas e digitalizadas para um computador para se quantificar os pixels coloridos.

7. Efeito das prostaglandinas no desenvolvimento folicular e ovulação

Ainda não é conhecido se as PGs possuem efeito direto sobre o desenvolvimento folicular antes do pico ovulatório de LH em bovinos, entretanto, sabe-se que a PGF2α aumenta a responsividade hipofisária ao hormônio liberador de gonadotropinas (GnRH) para secretar LH durante o período de pós-parto [Randel et al., 1996]. Por outro lado, vários estudos [Murdoch et al., 1993; Weems et al., 2006; Fortune et al., 2009] têm indicado que as PGs produzidas pelo folículo ovulatório são essencias para a ovulação.

A ovulação envolve uma complexa cascata de mudanças hormonais e moleculares no folículo pré-ovulatório. Na ovelha e na vaca, a ovulação ocorre por volta de 24-30 horas após o pico ovulatório de LH [Murdoch, 1985; Algire et al., 1992; Acosta et al., 2000; Bridges et al., 2006]. Em ovinos, ocorre aumento na expressão de COX-2, PGF2α e PGE2 após 8 horas do pico de LH, se extendendo por mais 4 horas até decrescer por volta de 16-20 horas após o

pico de LH [Murdoch et al., 1986; 1993). Na vaca, após o pico gonadotrópico pode ser observado aumento inicial, porém modesto, na secreção de PGs pelo folículo ovulatório [Bridges et al., 2006]. Nos momentos próximos à ovulação, a expressão de COX-2 nas células da granulosa e a concentração de PGs no líquido folicular atingem seus níveis máximos [Bridges et al., 2006].

Evidências que as PGs são reguladores essenciais no processo ovulatório têm sido acumuladas ao longo dos últimos 30 anos. Diversos estudos têm reportado que o uso de inibidores da COX, próximo ao pico de LH, reduz a síntese de PGs e está associado com a inibição da ovulação em mamíferos. Na rata, comundonga, coelha, ovelha, porca, vaca e em primatas, a administração de endometacina bloqueia a ovulação [O'Grady et al., 1972; Ainsworth et al., 1979; Downey et al., 1980; De Silva & Reeves, 1985; Killick & Elstein, 1987; Watson & Sertich, 1991]. Na égua, a intensa administração no período peri-ovulatório de outro DANE, o FM, também inibe eficientemente a ovulação, levando ao desenvolvimento da síndrome do folículo anovulatório hemorrágico [Cuervo-Arango, 2011a; Cuervo-Arango et al., 2011b; Ginther et al., 2011e].

As funções atribuídas às PGs no processo de ovulação incluem vasodilatação tecidual e alterações na região apical do folículo préovulatório [Murdoch et al., 1986; Priddy & Killick, 1993; Murdoch, 1996]. As PGs possuem papel fundamental no processo de remodelamento da matriz extracelular [Curry & Osteen, 2001]. Adicionalmente, foi reportado [Barreta et al., 2008; Siqueira, 2011] o envolvimento das PGs como agentes intermediários entre o pico de LH e o reinício da meiose em oócitos bovinos.

As colagenases aumentam em torno de 20 horas após o pico de LH e se mantêm elevadas até a ruptura do folículo ovulatório, e assim desempenham importante função no processo de ruptura da parede folicular [Murdoch & McCormick, 1991, Murdoch, 2000]. A inibição da PGs pelo tratamento com endometacina reduz tanto os níveis de colagenases, 24 horás após o pico de LH, como a infiltração leucocitária na parede folicular [Murdoch et al., 1993; Murdoch and McCormick, 1991]. Neste contexto, a infiltração leucocitária pode ser resultado dos quimiotáticos produzidos pelas colagenases e fundamental para ruptura folicular [Murdoch and McCormick, 1989, 1991, 1993]. O arranjo das organelas das células da granulosa, os cromossomos das células

foliculares, e o potencial de membrana celular no momento da ovulação são alterados pelas PGs, resultando em processo de necrose local [Ackerman & Murdoch, 1993; Murdoch, 2000; Murdoch & Lund, 1999; Murdoch & McDonnel, 2002].

As células da granulosa são responsáveis por grande parte da síntese dessas PGs nos folículos periovulatórios, no entanto, as células da teca e do cumulus também possuem capacidade de síntese de PGs em pequenas quantidades [Bridges et al., 2006]. Diversos subtipos de receptores para PGs estão presentes no folículo ovulatório e apresentam um complexo padrão de expressão durante todo o período préovulatório em bovinos, o que dificulta a identificação de suas funções [Bridges & Fortune, 2007].

As funções específicas da PGE2 e da PGF2α durante o processo ovulatório e de luteinização não são totalmente entendidas. Em camundongas, enquanto a deleção dos receptores para PGF2α não parece afetar a fertilidade, a ausência de receptores para PGE2 diminui o tamanho da ninhada por diminuir a taxa de ovulação [Sugimoto et al., 1997; Kennedy et al., 1999]. No entanto, em ruminantes a PGF2α *in vivo* parece possuir papel central no processo ovulatório [Murdoch et al., 1986]. Além disso, Barreta et al. (2008) demonstraram que a adição tanto de PGE2 quanto de PGF2α ao meio de cultivo induz a maturação nuclear de oócitos bovinos. Em ovelhas, injeções intrafoliculares de LH aumentam a expressão de PGE2, mas não de a PGF2α nas células da granulosa, entretanto, nas células da teca ocorre o aumento tanto de PGE2 como PGF2α [Murdoch and McCormick, 1991; Murdoch et al., 1993]. Adicionalmente, a inibição da ovulação pela endometacina em ovelhas pode ser revertida pelo tratamento com PGE2 ou PGF2α [Murdoch and McCormick, 1991; Murdoch et al., 1993].

Esses resultados em conjunto indicam o papel central e indispensável das PGs no processo de ovulação em mamíferos. Entretanto, os mecanismos intracelulares estimulados especificamente pela PGE2 e PGF2α que levam à ovulação e a retomada da maturação nuclear do oócito ainda são pouco compreendidos.

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CAPÍTULO 2- STUDY 1

ESTUDO ENVIADO PARA PUBLICAÇÃO

Effects of inhibition of prostaglandin F2α biosynthesis during preluteolysis and luteolysis in heifers

G. Pugliesi[a,b,c], H.K. Shrestha[a], M.A. Hannan[a], G.R. Carvalho[c],

M.A. Beg[b], O.J. Ginther[a,b]

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Effects of inhibition of prostaglandin F2α biosynthesis during preluteolysis and luteolysis in heifers

(Efeitos da inibição da síntese de prostaglandina F2α durante a pré-luteólise e luteólise em novilhas)

G. Pugliesi[a,b,c], H.K. Shrestha[a], M.A. Hannan[a], G.R. Carvalho[c], M.A. Beg[b], O.J. Ginther[a,b]

[a]Eutheria Foundation, Cross Plains, Wisconsin 53528, Estados Unidos da América

[b]Department of Pathobiological Sciences, University of Wisconsin, Madison, Wisconsin 53706, Estados Unidos da América

[c]Departamento de Zootecnia, Universidade Federal de Viçosa,Viçosa, Minas Gerais, Brasil

RESUMO

Flunexine meglumine (FM; 2,5 mg/kg) foi administrado em novilhas no 16º dia pós-ovulação, a cada 8 horas, por 24 horas (primeiro tratamento = Hora 0), objetivando inibir a síntese de prostaglandina F2α (PGF2α), baseando-se na mensuração da concentração plasmática do metabólito da PGF2α (PGFM). Amostras de sangue foram colhidas em intervalos de 8 horas do 15º ao 18º dia pós-ovulação em um grupo veículo (controle) e um grupo tratado com FM (n = 16/grupo). Adicionalmente, amostras de sangue foram colhidas de hora em hora da Hora −2 a 28 em 10 novilhas de cada grupo. As novilhas foram subdivididas naquelas que estavam em pré-luteólise e luteólise na Hora 0 baseado nas concentrações de progesterona (P4) de 8 em 8 horas. As concentrações de PGFM foram reduzidas (P < 0,05) pelo tratamento com FM em cada subgrupo. Para o subgrupo pré-luteolítico, o primeiro decréscimo (P < 0,05) nas concentrações de P4 ocorreu, respectivamente, nas Horas 24 e 40 nos grupos veículo e FM. As concentrações plasmáticas de P4 32 e 40 horas

após o início da luteólise no subgrupo luteolítico foram maiores (P < 0,05) no grupo FM. As concentrações de PGFM no pico do pulso de PGFM no grupo FM foram maiores (P < 0,05) no subgrupo luteolítico do que no subgrupo pré-luteolítico. O pico do pulso de PGFM ocorreu mais frequentemente (P < 0,001) na mesma hora que o pico de LH do que na mesma hora do nadir final da flutuação de LH. Em conclusão, a redução na proeminência dos pulsos de PGFM durante a luteólise retardou o fim da luteólise, e o tratamento com FM inibiu a produção de PGFM mais durante a pré-luteólise do que durante a luteólise.
Palavras-chaves: LH; Luteólise; Novilhas; PGFM; Progesterona

ABSTRACT

Flunixin meglumine (FM; 2.5 mg/kg) was given to heifers at three 8-h intervals, 16 d after ovulation (first treatment = Hour 0) to inhibit the synthesis of prostaglandin F2α (PGF2α), based on plasma concentrations of a PGF2α metabolite (PGFM). Blood samples were collected at 8-h intervals from 15 to 18 d in a vehicle (control) and FM group (n = 16/group). Hourly samples were collected from Hours –2 to 28 in 10 heifers in each group. Heifers that were in preluteolysis or luteolysis at Hour 0 based on plasma progesterone (P4) concentrations at 8-h intervals were partitioned into subgroups. Concentration of PGFM was reduced (P < 0.05) by FM treatment in each subgroup. For the preluteolytic subgroup, the first decrease (P < 0.05) in P4 concentration after Hour 0 occurred at Hours 24 and 40 in the vehicle and FM groups, respectively. Plasma P4 concentrations 32 and 40 h after the beginning of luteolysis in the luteolytic subgroup were greater (P < 0.05) in the FM group. Concentration at the peak of a PGFM pulse in the FM group was greater (P < 0.05) in the luteolytic than in the preluteolytic subgroup. The peak of a PGFM pulse occurred more frequently (P<0.001) at the same hour as the peak of an LH fluctuation than at the ending nadir of an LH fluctuation. In conclusion, a reduction in prominence of PGFM pulses during luteolysis delayed completion of luteolysis, and treatment with FM inhibited PGFM production more during preluteolysis than during luteolysis.
Keywords: Heifers; LH; Luteolysis; PGFM pulses; Progesterone

1. Introduction

The secretion of prostaglandin $F_{2\alpha}$ (PGF2α) from the uterus induces luteolysis in several species, including cattle [1,2]. Secretion of PGF2α is based frequently on assay of plasma concentrations of 13,14-dihydro-15-keto- PGF2α (PGFM), a metabolite of PGF2α [3]. The circulatory half-life of PGFM (approximately 8 min) is much longer than for PGF2α [4], and a sampling interval of 1 h has been recommended for detection of PGFM pulses in cattle [3]. Pulses of PGFM are 3 to 6 h in duration, with 4 to 7 pulses occurring during 2 to 3 d [3,5]. Sequential pulses of PGF2α, as determined by PGFM concentrations, are required to induce complete luteolysis in cattle [6], horses [7], and sheep [8]. Based on temporal relationships, PGF2α has a negative effect on progesterone (P4) concentration, which is balanced against a positive effect of LH pulses on P4 toward the end of the preluteolytic period and during the early luteolytic period in heifers [9,10].

Pulses of PGFM occur during the preluteolytic, luteolytic, and postluteolytic periods in cattle; these pulses have been characterized for each period [11,12]. During the luteolytic period, pulses have greater PGFM concentrations for the peak and amplitude than during the preluteolytic period. During the ascending portion of the last PGFM pulse of the preluteolytic period, P4 decreases to a low concentration at the peak of the PGFM pulse, followed by a P4 increase (rebound) to the prepulse concentration [9,13]. The complete rebound in P4 after the peak of a spontaneous PGFM pulse during the preluteolytic period is similar to the rebound that occurs during an estradiol-induced pulse of PGFM 14 d after ovulation [14,15], and also is similar to the complete rebound 1 or 2 h after the beginning of PGF2α infusion to simulate a PGFM pulse, 9 d after ovulation [16,17]. During early luteolysis, P4 also decreases during the ascending portion of the PGFM pulse, but the rebound does not return to the prepulse concentration [11,12,18]. Later during luteolysis, the rebound does not occur. The peak of the last PGFM pulse of preluteolysis occurred 4 h (range, 2 to 8 h) before the beginning of luteolysis (transitional hour) and the first pulse during luteolysis occurred 9 h after transition [9,13]. Therefore, the last PGFM pulse during preluteolysis initiated the subsequent onset of luteolysis.

The biosynthesis of PGF2α in the endometrium involves release of arachidonic acid (AA) from membrane phospholipids, catalyzed by the hormone-responsive enzyme cytosolic phospholipase A_2 [19]. Free AA is converted to prostaglandin H_2 (PGH_2) by cyclooxygenase enzyme (COX) in an oxidative conversion [20,21]. The PGH_2 is a precursor of PGF2α [22]. The conversion of AA is generally considered the rate-limiting step in prostaglandin production. Nonsteroidal anti-inflammatory drugs (NSAIDs [22]) are potent inhibitors of COX by competing with AA for active binding sites on the COX molecule. Flunixin meglumine (FM), one of the commonly used NSAIDs [23], acts on two COX isomers (COX 1 and COX 2 [24]), and blocks PGF2α synthesis [23]. After a single FM injection (2.2 mg/kg body weight) in ovariectomized cows, plasma PGFM concentrations decreased within 30 min and the effect continued for approximately 6 h [25]. Furthermore, PGFM concentrations were suppressed after twice daily im treatment with FM (1 g) for 5 d in postpartum cattle [26]. Intensive daily treatments of FM for 7 to 10 d, including the luteolytic period in cattle [23,25,27] and when administered twice daily in sheep [28], prolonged the life of the corpus luteum (CL) and lengthened the estrous cycle. However, the effects of short-term treatment with FM on PGFM pulses during the preluteolytic and luteolytic periods and on luteolysis have apparently not been reported.

The purpose of Experiment 1 was to determine a minimal dose of FM that effectively blocked PGF2α biosynthesis during the expected period of luteolysis in cattle. In Experiment 2, the effects of short-term FM treatment on PGFM pulses during the preluteolytic and luteolytic periods were characterized. The objectives were to: 1) compare between preluteolysis and luteolysis the effectiveness of FM for decreasing PGFM concentrations and the prominence of PGFM pulses; 2) study the effect of PGF2α pulses during preluteolysis on the onset of luteolysis; 3) determine the relationship between the prominence of PGFM pulses during luteolysis and the length of the luteolytic period; and 4) evaluate intrapulse relationships among episodes of PGFM, P4, and LH in heifers given a vehicle (control) or FM. The goal was to obtain information on the luteal response to short-term inhibition of PGF2α production, so that the PGF2α-inhibition approach would be available for testing physiologically oriented hypotheses.

2. Materials and methods

2.1. Animals

Dairy heifers (Holsteins) aged 20 to 22 mo and weighing 578 ± 16 kg (range, 500 to 620 kg) were used. Heifers were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research. Heifers were selected with docile temperament and no apparent abnormalities of the reproductive tract, as determined by transrectal ultrasonography [29]. If more than one CL was present, or the single CL was considered undersized (< 3 cm^2) on day 15 after ovulation, the heifer was not used. Heifers were kept under natural light in an open-sided shelter and outdoor paddock and were maintained by *ad libitum* access to a mixture of alfalfa and grass hay, water, and trace-mineralized salt, with supplementary feeding of ground corn (2 kg/heifer/d). Heifers remained healthy and in good body condition throughout each experiment. The day of ovulation was determined by daily transrectal ultrasonographic examinations [29]. These experiments were performed during January to March in the northern temperate zone.

2.2. Experiment 1

Heifers were assigned randomly by replicate to one control group and three FM-treated groups (n = 4 heifers/group). At 16 d after ovulation, heifers in the FM-treated groups were given a single im administration of FM (FluMeglumine®, 50 mg/mL, Phoenix Pharmaceutical, Inc., St. Joseph, MO, USA) using three dose groups. The three FM-treated groups were FM-0.5 (0.5 g FM), FM-1.0 (1.0 g FM), and FM-1.5 (1.5 g FM). The doses of FM used in each group (0.5, 1.0, and 1.5 g/heifer) corresponded to mean doses of 0.9, 1.7, and 2.5 mg/kg body weight, as determined retrospectively. The hour of FM treatment or the corresponding hour in controls was designated Hour 0. Blood samples were collected hourly by venipuncture from the coccygeal vein during Hours –2 to 10. Plasma samples were assayed for PGFM to determine the minimal effective dose of FM. The mean overall PGFM concentration and

transient increases in PGFM after Hour 0 with a peak >100 pg/mL were used as indicators of the effectiveness of the FM doses.

2.3. Experiment 2

Heifers were assigned to a vehicle group and an FM-treated group (n = 16/group; Fig. 1). Heifers in the vehicle group were given 0.05 mL/kg body weight of physiologic saline im every 8 h, whereas those in the FM group were given 2.5 mg/kg body weight of FM im every 8 h. The dose of FM and interval between treatments were based on the finding in Experiment 1 that a single FM treatment of 2.5 mg/kg inhibited transient PGFM increases for 9 h. The treatments were given 16 d after ovulation, based on functional regression of the CL at 15, 16, or 17 d after ovulation in heifers [5,12,30]. Treatments were given at 08:00 (Hour 0), 16:00 (Hour 8), and 00:00 (Hour 16).

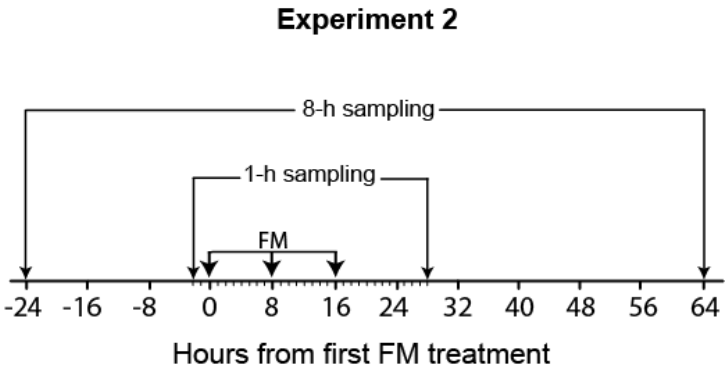

Fig. 1. Diagram of the protocol for Experiment 2. Flunixin meglumine (FM; 2.5 mg/kg body weight) was given to heifers at three 8-h intervals, 16 d after ovulation (first treatment = Hour 0). The 8-h blood sampling was done from 08:00 on day 15 (Hour –24) until 00:00 18 d (Hour 64) after ovulation (n = 16). The hourly sampling was done during 30 h for Hours –2 to 28 (n = 10).

A blood sample (10 mL) was taken from the coccygeal vein every 8 h, beginning at 08:00 on 15 d until 00:00 on 18 d after ovulation. The 8-h samples were used to determine the beginning and end of luteolysis in each heifer. Transition between preluteolytic and luteolytic periods was defined as occurring at the sample before P4 concentrations decreased ≥ 1 ng/mL between 8-h intervals, and was followed by a progressive decrease in concentrations until the end of luteolysis. The end of the preluteolytic period and beginning of the luteolytic period (transition) was at a common 8-h sample. The end of luteolysis

was defined by the 8-h sample before P4 decreased to < 1 ng/mL [5,31]. The length of luteolysis was the interval between the beginning and end of the luteolytic period. All 16 heifers/group were used for study of P4 at 8-h intervals. For further data analyses, the heifers were subgrouped into those that were in the preluteolytic or luteolytic periods at the first treatment (Hour 0).

In addition to the 8-h samples, blood samples were taken each hour for 30 h from Hour –2 to Hour 28, in 10 heifers/group, to study hourly concentration changes, and the number and location of episodes of PGFM, P4, and LH. Hourly blood samples were collected through an indwelling catheter, placed into a jugular vein and secured as described [32]. The catheter was inserted 15 h before collection of the first hourly sample. Heifers had *ad libitum* access to water and hay between collections of blood samples.

Hourly concentrations of PGFM, P4, and LH were analyzed initially without regard to the location of hormone episodes from Hours 1 to 28, with Hour 1 corresponding to the beginning of inhibition of PGFM concentrations by FM treatment. A transient hormone increase and decrease (fluctuation) in individual heifers that encompassed at least four values (PGFM) or three values (P4 and LH) including nadirs with a progressive increase on the ascending portion and progressive decrease on the descending portion were evaluated. The coefficient of variation (CV) of hormone concentrations in the fluctuation and the intra-assay CV were used to distinguish extraneous fluctuations from episodes of PGFM, P4, and LH as previously described [5,33,34]. When the CV of the values composing a fluctuation of PGFM and LH was at least three times the mean intra-assay CV, the fluctuation was defined as a pulse. These definitions were based on reports that episodes of PGFM [3,13,35] and LH [9,35] can be detected by sampling blood at 1-h intervals. However, the use of three times the intra-assay CV was not effective in identifying P4 pulses. Therefore, fluctuations of P4 with a CV at least one time greater than the intra-assay CV were defined only as fluctuations. Episodes of PGFM, P4, and LH with a peak near the beginning or end of the hourly-sampling session were included in the CV analyses only if they were preceded or followed, respectively, by at least one lower value, but the lower value was not used to calculate nadir concentrations. Nadirs 1 and 2 were defined as the nadirs at the beginning and end of a pulse

or fluctuation. Amplitude was the difference in concentration between Nadir 1 and the peak.

The hour of the peak (0 h) of a CV-identified PGFM or LH pulse was used for centralizing and comparing the intrapulse concentrations between the two groups (vehicle and FM). Comparisons between groups involved the overall mean concentration of PGFM, P4, and LH from Hour 1 to Hour 28; number of CV-identified pulses (PGFM and LH) and fluctuations (P4); concentration of each hormone at each hour; concentrations during episodes (peak, amplitude, Nadir 1, and Nadir 2); length of intervals between episodic events; and intrapulse concentrations from –2 to 2 h centralized to the peak (0 h) of PGFM or LH pulses. Separate determinations for the preluteolytic and luteolytic subgroups within the vehicle and FM groups were made for the following: 1) frequency of occurrence of the peak of a CV-identified LH pulse at the peak of a P4 fluctuation and 2) frequency of occurrence of the peak of a CV-identified PGFM pulse at the peak, Nadir 1, or Nadir 2 of an LH fluctuation, including those that did not meet the definition of a CV-identified LH pulse.

2.4. Hormone assays

Blood samples were immediately placed in ice water for 10 min after collection, followed by centrifuging (2 000 X *g* for 10 min). The plasma was decanted and stored (–20 ºC) until assayed. Plasma samples were assayed for PGFM by an ELISA that was developed and validated in our laboratory for use with bovine plasma [12]. Plasma P4 concentrations were assayed with a solid-phase radioimmunoassay (RIA) kit containing antibody-coated tubes and ^{125}I-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA). The procedure has been validated and described [5] for bovine plasma in our laboratory. Plasma concentrations of LH were determined by RIA as described [36] and modified [37] for bovine plasma in our laboratory. For PGFM, the intra- and interassay CV and sensitivity were 7.0%, 18.7%, and 14.4 pg/mL, respectively in Experiment 1, and were 12.0%, 18.4%, and 10.6 pg/mL, respectively, in Experiment 2. In the latter experiment, the intraassay CV and sensitivity for P4, were 9.6% and 0.05 ng/mL, respectively, and for LH were 6.6%, and 0.02 ng/mL.

2.5. Statistical analyses

Outlying observations greater than three interquartile ranges from the mean were not used in the statistical analyses (PROC UNIVARIATE; SAS online Doc 9.1.3 SAS Institute Inc., Cary, NC, USA). This was indicated only for one observation from the 8-h P4 data. Data were examined for normality using the Shapiro-Wilk test. Data that were not normally distributed were transformed to natural logarithms or ranks. For each experiment and for each hormone, the main effect of group and hour and the interaction were analyzed. The SAS PROC MIXED (Version 9.2; SAS Institute) was used for analyses, with a REPEATED statement to account for autocorrelation between sequential measurements. Unpaired Student's *t*-tests were used to locate differences between two groups within an hour. The Least Significant Difference (LSD) test was used for comparisons between hours within a group. When there was an apparent disparity between groups at Hour 0, the percentage change from Hour 0 or a covariance analysis with Hour 0 as the covariate was used. Discrete or single-point data for characteristics and intervals for PGFM and LH pulses and P4 fluctuations were analyzed for differences between groups by unpaired Student's *t*-tests. Hormone concentrations during CV-identified PGFM and LH pulses were compared between groups in an hour-by-group analysis using the peak of the pulse of one hormone (0 h) as a centralized reference point for another hormone. The LSD test was used to locate differences among hours within each group. The PGFM pulses were analyzed for main effect of group, subgroup (preluteolytic and luteolytic), and hour and for the interaction among these three factors. A chi-square test was used for the frequency of occurrence of the peak of LH pulses at the peak of P4 fluctuations and for the peak of PGFM pulses at the peak, Nadir 1 or Nadir 2 of LH fluctuations. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $P > 0.05$ to $P \leq 0.1$ indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise indicated.

3. Results

3.1. Experiment 1

There was considerable disparity among groups in the mean PGFM concentration at Hour 0. Therefore, data were converted to the percentage change from Hour 0 in each heifer. The percentage change in PGFM concentrations for Hours 1 to 10 had only a group effect (Fig. 2). The group effect reflected the following percentage change in PGFM concentration averaged over Hours 1 to 10: control (254 ± 253 %^{a}), FM-0.5 (47 ± 79 %^{ab}), FM-1.0 (–49 ± 11 %^{b}) and FM-1.5 (–80 ± 4 %^{c}); means without a common superscript were different ($P < 0.05$). The corresponding PGFM concentrations averaged over Hours 1 to 10 were 88.8 ± 50.9, 53.5 ± 23.1, 33.9 ± 20.9 and 24.2 ± 6.0 pg/mL in the four groups, respectively. For heifers with a transient PGFM increase (peak >100 pg/mL) after FM treatment, the distribution for the number of heifers, hours of each peak concentration, and concentration at the peak were as follows: controls, two heifers, Hours 4 and 6, 117.6 pg/mL and 196.3 pg/mL; FM-0.5, one heifer, Hour 6, 736.7 pg/mL; FM-1.0, one heifer, Hour 3, 119.5 pg/mL; and FM-1.5, one heifer, Hour 9, 123.0 pg/mL.

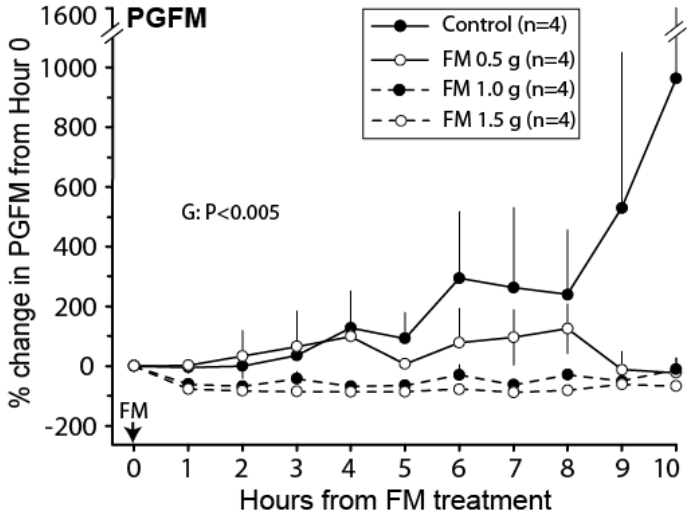

Fig. 2. Mean ± SEM percentage change for PGFM concentration from treatment (Hour 0) in controls and three groups treated with a single injection of the indicated dose of flunixin meglumine (FM; 4 heifers/group) 16 d after ovulation. Main effect of group (G) was significant, but neither hour effect nor interaction were significant. Experiment 1.

3.2. Experiment 2

For analyses that involved 8-h sampling intervals, six of the 16 heifers in the vehicle group and four of the 16 heifers in the FM group were omitted because luteolysis was complete by the hour of first treatment (Hour 0) 16 d after ovulation or had not begun by the end of the blood sampling at 18 d. For the remaining 10 heifers in the vehicle group, six were in the preluteolytic period and four were in the luteolytic period at Hour 0. For the remaining 12 heifers in the FM group, seven were in the preluteolytic period and five were in the luteolytic period. For the 10 heifers/group with blood collection every hour (Hours –2 to 28), one heifer in the vehicle group was omitted because luteolysis was complete by Hour 0. In the vehicle group, seven were in the preluteolytic period and two were in the luteolytic period at Hour 0 and were assigned to the subgroups. In the FM group, seven were in the preluteolytic period and three were in the luteolytic period.

The probabilities for a group effect, hour effect, and a group-by-hour interaction for the factorial analyses are given in the figures and the probabilities for differences in discrete end points are given in the table or text. The intervals (days) from pretreatment ovulation to the beginning of luteolysis, to the end of luteolysis, and to posttreatment ovulation were not different ($P > 0.1$) between groups (data not shown). Based on 8-h sampling, the intervals from beginning to end of luteolysis in the subgroups of the vehicle and FM groups, respectively, were as follows: preluteolytic subgroup, 24.0 ± 2.9 h and 29.7± 2.9 h ($P < 0.1$); luteolytic subgroup, 16.0 ± 4.6 h and 27.2 ± 3.2 h ($P < 0.03$).

For heifers that were in the preluteolytic subgroup at the hour of the first treatment (Hour 0), P4 concentrations in the samples collected every 8 h had only a significant hour effect, even when a covariance analysis was used (Fig. 3). Although there was no interaction, concentration approached being greater ($P < 0.09$) in the FM group than in vehicle group at Hour 24. When each group in the preluteolytic subgroup was analyzed separately, the first decrease ($P < 0.05$) in P4 concentrations occurred between Hours 8 and 16 in the vehicle group and between Hours 0 and 40 in the FM group. The first decrease ($P < 0.05$) in P4 concentration from Hour 0 occurred at Hours 24 and 40 in the vehicle and FM groups, respectively. The intervals from Hour 0 to the hour of

the beginning of luteolysis in the vehicle and FM groups, respectively, were 24 ± 6.9 h and 32.0 ± 4.3 h (P > 0.1). The interval from Hour 0 to the end of luteolysis in the preluteolytic subgroup of the vehicle and FM groups, respectively, were as follows: 48.0 ± 8.8 h and 61.7 ± 4.2 h (P < 0.08). Two of six heifers in the vehicle group and none of seven heifers in the FM group (P < 0.1) began luteolysis on the day of the three treatments. For heifers that were in the luteolytic subgroup, the P4 concentrations in samples collected every 8 h centralized to the beginning of luteolysis had an hour effect and an interaction of group by hour (Fig. 4). Concentrations approached being greater at Hours 16 and 24 and were greater at Hours 32 and 40 in the FM group than in the vehicle group.

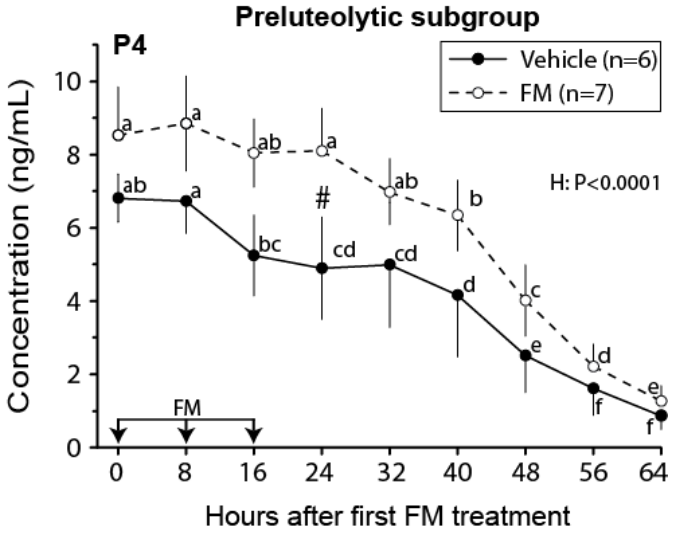

Fig. 3. Mean ± SEM progesterone (P4) concentration at 8-h intervals in a vehicle group and a group treated with flunixin meglumine (FM; 2.5 mg/kg body weight) every 8 h for 16 d after ovulation. Heifers were in the preluteolytic subgroup at first treatment (Hour 0). Main effects of group (G) and hour (H) and interaction that were significant or approached significance are shown. Hour of an approached difference (P < 0.09) in P4 between groups is indicated by a hatch mark (#). Experiment 2.
[a-f] Within a group, means without a common letter differed (P < 0.05).

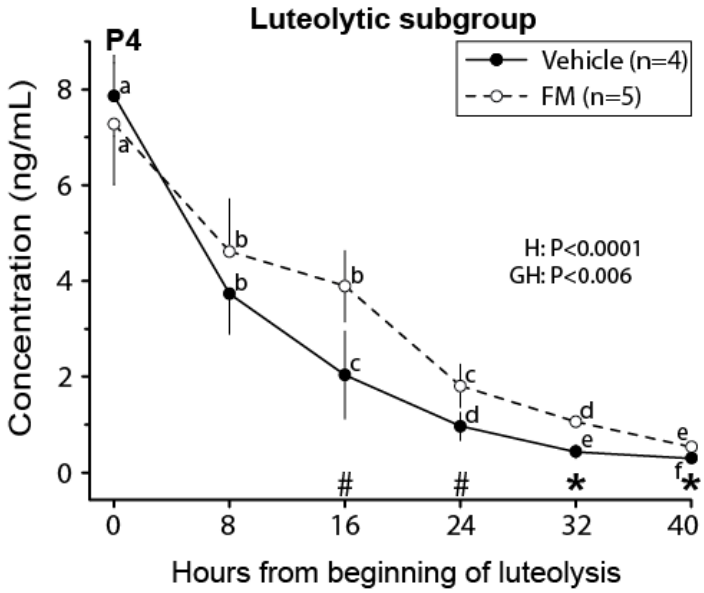

Fig. 4. Mean ± SEM progesterone (P4) concentration at 8-h intervals in a vehicle group and a group treated with flunixin meglumine (FM; 2.5 mg/kg body weight) every 8 h for 16 d after ovulation. Heifers were in the luteolytic period at first treatment. Main effects of group (G) and hour (H) and interaction (GH) that were significant or approached significance are shown. Hours of a difference ($P < 0.05$) or approaching difference ($P < 0.1$) in P4 between groups are indicated, respectively, by an asterisk (*) or a hatch mark (#). Experiment 2.
[a-f] Within a group, means without a common letter differed ($P < 0.05$).

Concentrations of PGFM, P4, and LH for the hourly intervals after the beginning of PGFM inhibition (Hours 1 to 28) in heifers that were in preluteolytic subgroup at Hour 0 are shown (Fig. 5). Only PGFM had a significant group effect, owing to a lower mean PGFM concentration in the FM group than in the vehicle group (Table 1). All hormones had a significant or approaching significant effect of hour. None of the hormones had an interaction of group by hour. When analyzed separately for each group, concentrations of P4 were lower than for Hour 0 in the vehicle group for 14 of 28 hours. There were no differences among hours for the FM group (Fig. 5).

Table 1.Mean ± SEM characteristics of plasma PGFM, progesterone (P4), and LH concentrations during 28-h sessions of hourly blood sampling for the preluteolytic subgroup[a] in a vehicle group and a group treated with flunixin meglumine (FM; 2.5 mg/kg body weight) every 8 h on day 16 after ovulation. Experiment 2.

End point	PGFM			P4			LH		
	Vehicle		FM	Vehicle		FM	Vehicle		FM
No. heifers[a]	7		7	7		7	7		7
Per 28-h sampling session:[b]									
No. episodes	2.3 ± 0.6		1.6 ± 0.6	5.7 ± 0.9	#	7.1 ± 0.4	6.3 ± 0.4	*	8.1 ± 0.7
Mean concentration[c]	104.1 ± 39.7	*	17.8 ± 4.6	7.6 ± 1.1		10.0 ± 0.9	0.28 ± 0.05		0.22 ± 0.02
Episode concentrations[c]									
Peak	308.8 ± 63.5	*	43.7 ± 7.3	9.6 ± 0.6	*	11.0 ± 0.5	0.49 ± 0.03	*	0.38 ± 0.02
Nadir 1[d]	90.3 ± 25.1	*	14.3 ± 2.0	6.9 ± 0.4	*	8.1 ± 0.3	0.15 ± 0.02		0.14 ± 0.01
Nadir 2[d]	74.9 ± 18.4	*	12.9 ± 3.2	6.5 ± 0.5	*	7.9 ± 0.3	0.17 ± 0.02	*	0.13 ± 0.01
Amplitude[e]	218.5 ± 52.9	*	29.5 ± 7.0	2.7 ± 0.3		2.9 ± 0.2	0.34 ± 0.03	*	0.24 ± 0.02
Episode intervals (h):									
Peak to peak	6.8 ± 0.9	*	3.5 ± 0.3	4.0 ± 0.3		4.0 ± 0.3	4.5 ± 0.3	*	3.3 ± 0.2
Nadir 1 to Nadir 2	4.9 ± 0.5	#	4.0 ± 0.4	3.6 ± 0.3	*	3.0 ± 0.2	3.8 ± 0.2	*	3.1 ± 0.2
Nadir 2 to Nadir 1	1.9 ± 1.1		0.0 ± 0.0	0.3 ± 0.1	*	1.0 ± 0.3	0.7 ± 0.2	*	0.2 ± 0.1

[a]Heifers were in the preluteolytic period at hour of first treatment and were defined as the preluteolytic subgroup.
[b]Blood sampling was done hourly for Hours –2 to 28 (Hour 0= first treatment), but data for Hours –2 to 0 were not included in the analyses.
[c]Concentrations (PGFM, pg/mL; P4 and LH, ng/mL)
[d]Nadirs 1 and 2 are nadirs at the beginning and the end of an episode (PGFM and LH pulse and P4 fluctuation), respectively.
[e]Amplitude is the difference in concentration between Nadir 1 and peak.
Means separated by an asterisk (*) or a hatch mark (#) within each hormone and end point (row) are different ($P < 0.05$) or approached a difference ($P < 0.1$), respectively, between groups.

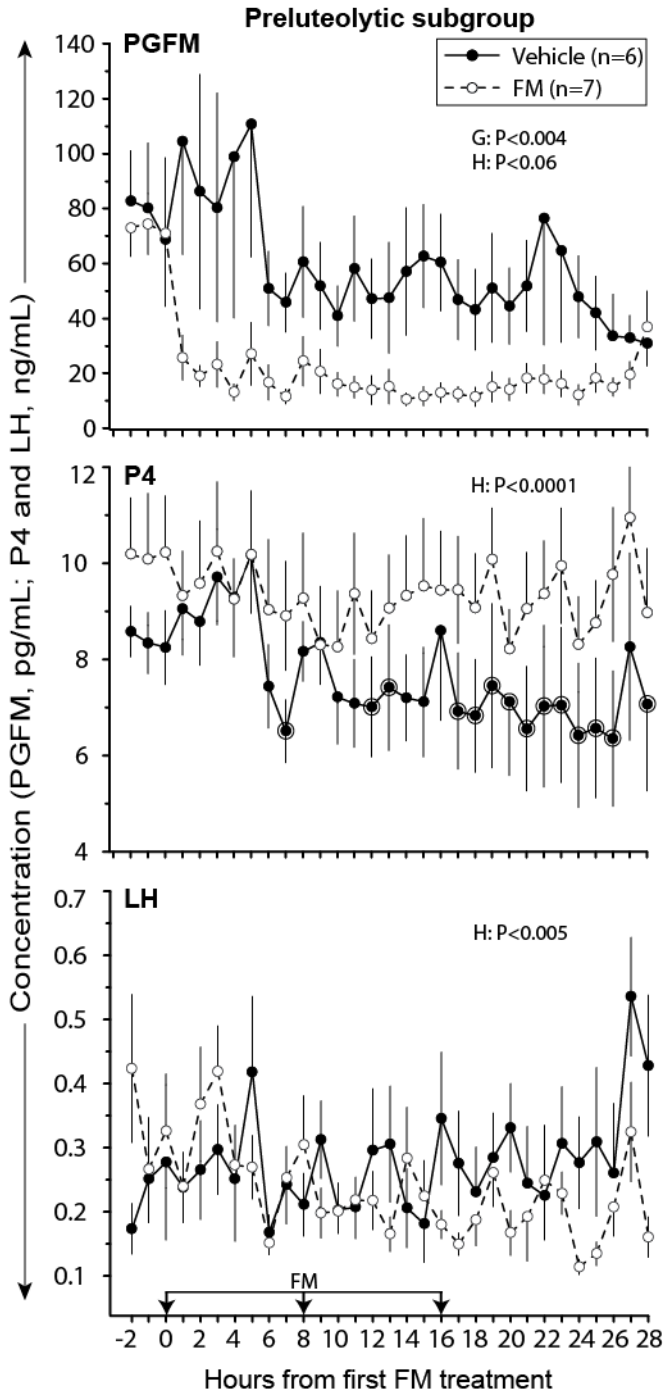

Fig. 5. Mean ± SEM concentrations of PGFM, progesterone (P4), and LH at hourly intervals in a vehicle group and a group treated with flunixin meglumine (FM; 2.5 mg/kg body weight) every 8 h for 16 d after ovulation. Heifers were in the preluteolytic subgroup at first treatment (Hour 0). The factorial analyses were done for Hours 1 to 28. Main effects of group (G) and hour (H) and the interaction that were significant or approached significance are shown. The hours of a lower P4 concentration ($P < 0.05$) from Hour 0 in the vehicle group is indicated by a circled mean. Experiment 2.

Differences between groups in the characteristics of episodes of PGFM, P4, and LH in the hourly data as determined by the CV methodology during

hourly sampling for heifers in the preluteolytic subgroup at Hour 0 are shown (Table 1). Hormone episodes for heifers that were in the luteolytic subgroup at Hour 0 were not characterized, owing to the limited number of heifers (vehicle group, n = 2; FM group, n = 3). For the preluteolytic subgroup, mean PGFM concentration/pulse and concentrations at the peak, Nadir 1, Nadir 2, and amplitude were lower ($P < 0.01$) and the interval from peak to peak was shorter ($P < 0.03$) in the FM group than in the vehicle group. The intrapulse concentration of PGFM centralized to the peak had significant effects for group and hour, but no effect of subgroup (preluteolytic and luteolytic; Fig. 6). The three-way interaction among group, hour, and subgroup was significant. Mean PGFM concentration in the FM group (39.4 ± 6.4 pg/mL) was lower than in the vehicle group (189.5 ± 18.9 pg/mL), greater in the luteolytic subgroup (57.1 ± 13.4 pg/mL) than in the preluteolytic subgroup (25.4 ± 3.0 pg/mL), and greater at the hour of the peaks of PGFM pulses, as expected from centralization to the peak. In addition, the PGFM concentration at the peak of a pulse in the FM group was greater ($P < 0.0001$) for the luteolytic subgroup (147.4 ± 44.2 pg/mL) than for the preluteolytic subgroup (43.7 ± 7.3 pg/mL), but concentration at the pulse peak in the vehicle group was not different between subgroups. In a separate factorial analysis of concentration at the peak of the PGFM pulses for the groups (vehicle and FM) and subgroups (preluteolytic and luteolytic), an interaction ($P < 0.05$) reflected a greater difference between groups in the preluteolytic subgroup than in the luteolytic subgroup. Thus, the three-way interaction represented primarily greater PGFM concentration at the peak of a pulse in the FM group during the luteolytic subgroup than during the preluteolytic subgroup without a difference between subgroups in the vehicle group. The differences between groups for each hour of each subgroup are shown (Fig. 6).

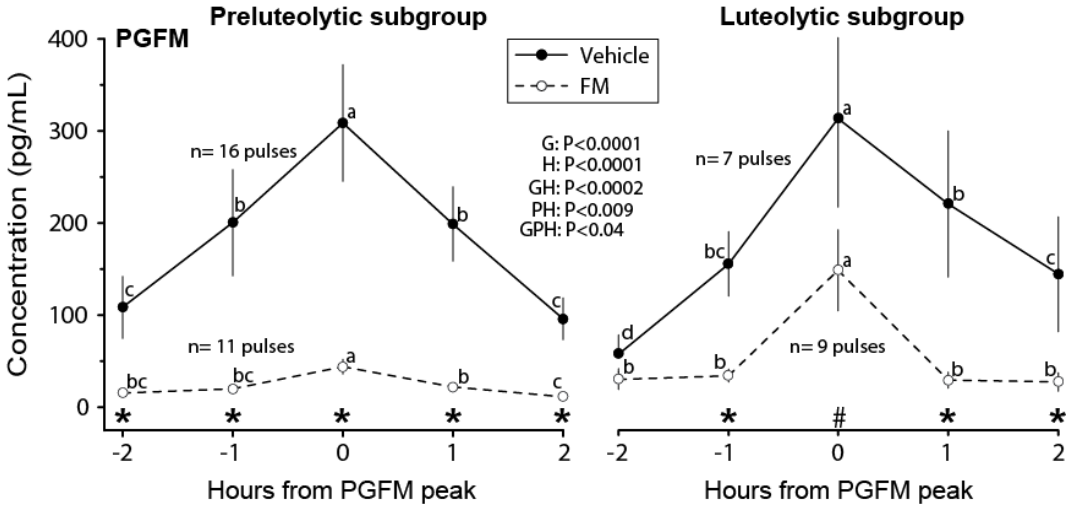

Fig. 6. Mean ± SEM concentrations in PGFM pulses in a vehicle group and a group treated with flunixin meglumine (FM; 2.5 mg/kg body weight) every 8 h for 16 d after ovulation. Heifers were in either preluteolytic subgroup or luteolytic subgroup at first treatment. The number of pulses for each group is shown. Main effects of group (G), period (P), and hour (H) and the interactions (GH, PH, and GPH) that were significant or approached significance are shown. Hours of a difference ($P < 0.05$) and an approaching difference ($P < 0.1$) in PGFM concentrations between groups are indicated, respectively, by an asterisk (*) and hatch mark (#). Experiment 2.
[a-d]Within a group, means without a common letter differed ($P < 0.05$).

The number of CV-identified P4 fluctuations/28 h approached being greater ($P < 0.09$) in the FM group than in the vehicle group (Table 1). For the P4 fluctuations, concentrations at the peak, Nadir 1, and Nadir 2 were greater; the interval between Nadir 1 and Nadir 2 was shorter; and the interval between Nadir 2 to Nadir 1 of the next fluctuation was longer in the FM group than in vehicle group. In the preluteolytic subgroup, changes in P4 concentration within the hours of a PGFM pulse were not detected for either group (data not shown). In the luteolytic subgroup, P4 concentration within a PGFM pulse had an hour effect and an interaction ($P < 0.0001$; not shown). The interaction represented primarily a decrease in P4 concentration from –2 to 2 h (0 h = peak) of a PGFM pulse in the vehicle group and a decrease between –2 to 0 h and maintenance between 0 to 2 h in the FM group.

Characteristics and concentrations of LH pulses in the preluteolytic subgroup are shown (Table 1). The number of LH pulses/28 h was greater; concentration at the peak, Nadir 2, and amplitude were lower; and the intervals from peak to peak, Nadir 1 to Nadir 2, and Nadir 2 to Nadir 1 were shorter in the FM group than in the vehicle group. In the preluteolytic subgroup, the peak of an LH pulse occurred at the same hour as the peak of a P4 fluctuation in 77%

(34 of 44) and 73% (41 of 56) of LH peaks in the vehicle and FM groups, respectively (P > 0.1). In the luteolytic subgroup, a P4 peak occurred at the same hour as an LH peak in 36% (4 of 11) and 42% (10 of 26) of LH peaks in the vehicle and FM groups, respectively (P > 0.1). Combined for the two groups, the frequency of synchronized P4 and LH peaks was greater (P < 0.0001) in the preluteolytic subgroup (75%) than in the luteolytic subgroup (38%).

The concentration profile for P4 centralized to the peak of LH pulses (–2 to 2 h; 0 h = pulse peak) in the preluteolytic subgroup had an hour effect averaged over the vehicle and FM groups, but not a group effect or interaction (Fig. 7). The hour effect represented an increase in P4 from Hours –2 to 0 h and a decrease from 0 to 1 h in each group. In the luteolytic subgroup, concentration of P4 centralized to the peak of LH pulses had an hour effect (P < 0.0001) and an interaction of group by hour (P < 0.03; not shown). The interaction represented a P4 decrease from –2 to –1 h and again at 1 to 2 h but not from –1 to 1 h in the vehicle group and a decrease between –2 and 1 h in the FM group.

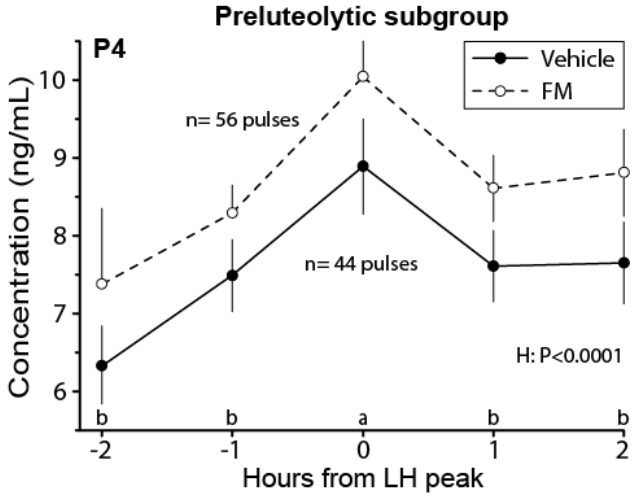

Fig. 7. Mean ± SEM concentrations of P4 within the hours of an LH pulse in a vehicle group and in a group treated with flunixin meglumine (FM; 2.5 mg/kg body weight) every 8 h for 16 d after ovulation. Heifers were in preluteolytic period at first treatment. The number of PGFM pulses for each group is shown. Main effects of group (G) and hour (H) and the interaction (GH) that were significant or approached significance are shown. Experiment 2.
[a,b]Combined means at each hour with a different letter on the X-axis differed (P < 0.05).

In the preluteolytic and luteolytic subgroups, the frequency of occurrence of the peak of a PGFM pulse at the components (peak, Nadir 1, or Nadir 2) of an LH fluctuation did not differ (P > 0.1) between the vehicle and FM groups (not shown). Combined for the two treatment groups, the frequency of

occurrence of the peak of a PGFM pulse at the peak, Nadir 1, and Nadir 2 of an LH fluctuation, respectively, were 52% (14 of 27), 30% (8 of 27), and 0% (0 of 27) for heifers that were in the preluteolytic subgroup and 19% (3 of 16), 19% (3 of 16), and 31% (5 of 16) for heifers that were in the luteolytic subgroup. The frequency of LH synchrony with PGFM components in the preluteolytic subgroup was different ($P < 0.004$) among components, but was not different in the luteolytic subgroup. Within the preluteolytic subgroup, the frequency of the peak of a PGFM pulse at the same hour as the peak of an LH fluctuation was greater ($P < 0.001$) than the frequency of the PGFM peak at Nadir 2 and approached being greater ($P < 0.1$) than the frequency at Nadir 1.

4. Discussion

The titration study (Experiment 1) indicated that a total dose per heifer of 0.5 g FM was ineffective in preventing PGF2α biosynthesis in cattle, whereas 1.0 g was partially effective, and 1.5 g (equivalent to 2.5 mg/kg) was most effective. In previous studies [23,25,27], using an FM dose of 2.2 mg/kg, the inhibition on PGFM production was most effective when FM was given four times/day. Inspection of individual PGFM profiles in Experiment 1 indicated that a single FM dose of 1.5 g/heifer lost effectiveness after 8 h in one heifer, as indicated by a transient increase in PGFM concentrations (> 100 pg/mL) at Hour 9 after FM treatment at Hour 0. For this reason, heifers were treated at 8-h intervals in Experiment 2.

Three treatments with FM at 8-h intervals beginning 16 d after ovulation in Experiment 2 reduced biosynthesis of PGF2α in heifers that were in the preluteolytic period at first treatment, as indicated by the lower PGFM concentrations in the FM group than in the vehicle group during each hour from Hours 1 to 25. In addition, FM treatment reduced the prominence but not the number of PGFM pulses in both the preluteolytic and luteolytic subgroups. The reduced pulse prominence in the FM group was indicated by lower PGFM concentrations averaged over all hours of a PGFM pulse (group effect in the factorial analysis) during each of the preluteolytic and luteolytic subgroups, and by the lower PGFM concentrations at the peak, Nadir 1, Nadir 2, and amplitude

of pulses in the FM group than in the vehicle group in the preluteolytic subgroup.

The FM was less effective during luteolysis than during preluteolysis, as indicated by the greater overall PGFM concentrations for pulses and greater concentration at the peak of a PGFM pulse in the luteolytic subgroup than in the preluteolytic subgroup of the FM group. In the vehicle group, the peak of PGFM pulses were not significantly different between subgroups. However, the reduced effectiveness of the FM treatment in the luteolytic subgroup may be related to greater PGF2α biosynthesis during the luteolytic period in untreated heifers, as indicated by greater concentration of PGFM at the peak and amplitude and a longer interval from peak to Nadir 2 during luteolysis than during preluteolysis [11,12]. The reduced inhibition of PGFM biosynthesis in the luteolytic subgroup compared to the preluteolytic subgroup in the FM group may have been due to more available AA during the luteolytic period, but this was not studied. In this regard, the AA released from membrane glycerophospholipids [38] competes with FM molecules to bind to the active site of COX enzyme [22]. Further study will be needed to determine if a higher dose will reduce the PGFM concentration to the sensitivity of the assay (11 pg/mL) and thereby more effectively interfere with luteolysis; however, side effects must also be considered.

Based on temporal relationships, the progressive decrease in P4 that followed the transitional hour was associated with the effects of the last PGFM pulse of the preluteolytic period [13]. In Experiment 2, two of six heifers in the vehicle group that were in the preluteolytic period at the hour of first treatment began luteolysis during the hourly sampling session. In one heifer, the peak of the last preluteolytic PGFM pulse occurred 4 h before the transitional hour, whereas the next pulse occurred after the transitional hour, similar to a previous report [13]. This phenomenon could not be examined in the second heifer, owing to an inadequate number of samples before the transitional hour. The nature of the effect of PGF2α pulses during preluteolysis on the onset of luteolysis is not known. In this regard, further study is needed on the effect of the preluteolytic PGF2α pulses on the enzymes, second messengers, and receptors [39,40] within luteal cells that subsequently lead to progressive reduction in the output of P4 production (luteolysis).

The delay in a P4 decrease after Hour 0 in the FM group compared to the vehicle group in heifers that were in the preluteolytic subgroup indicated an inhibition of PGF2α secretion during preluteolysis. However, all other related end points only approached significance, including the following differences in the FM group: 1) greater P4 concentration at Hour 24, 2) longer length of the luteolytic period, 3) longer interval from Hour 0 to the end of luteolysis, and 4) fewer heifers that began luteolysis during the day of the three treatments. The beginning of luteolysis on the day of the FM treatments in 2/6 heifers in the vehicle group and 0/7 heifers in the FM group in turn accounted for all of these tendencies. For these reasons, whether short-term blockage of PGF2α secretion during preluteolysis affected the time of the onset and the length of luteolysis was considered equivocal. A short-term effect of PGF2α inhibition during preluteolysis has not been previously reported. However, inhibition of PGF2α secretion in heifers that were in luteolysis at the hour of first FM treatment inhibited luteolysis, as indicated by the longer length of the luteolytic period and the greater P4 concentration during the later portion of luteolysis in the FM group. The interference with luteolysis after short-term FM treatment during luteolysis accounted for previous reports that FM administration for 7 to 10 d, including the expected period of luteolysis, postponed luteolysis and prolonged the estrous cycle [23,25,27].

Previous studies established that sequential episodic PGF2α secretion was necessary for complete luteolysis in several species (see Section 1). In Experiment 2, it was not determined whether continued sequential PGFM pulses were required during the luteolytic period for completion of luteolysis, owing to only partial reduction in the prominence of PGFM pulses by FM when luteolysis was underway at Hour 0. However, the reduced prominence of PGFM pulses in the luteolytic subgroup of the FM group compared to the vehicle group allowed evaluation of the effect of the prominence of PGFM pulses on luteolysis. The results indicated that the magnitude of sequential PGFM pulses affected the time required for luteolysis to be completed, based on the greater P4 concentrations at 32 and 40 h after the beginning of luteolysis in the FM group. The effect of greater prominence of PGF2α pulses on reducing the time required for completion of luteolysis was a novel observation. In this regard, intrauterine infusion of a higher dose of PGF2α resulted in more rapid

regression of the CL than a lower dose, but the higher dose was considered pharmacologic [6].

For heifers in the preluteolytic subgroup at Hour 0, the greater LH concentration at various portions of an LH pulse (Table 1) and the lesser number of LH pulses in the vehicle group than in the FM group may reflect the decrease in P4 concentration during hourly sessions in the vehicle group. In this regard, increased LH concentrations and LH pulsatility after the transition from the preluteolytic period to the luteolytic period has been reported [9]. The LH increase may represent the removal of the inhibitory action of P4 on LH release from hypophyseal cells, as demonstrated in vitro [41]. An alternative interpretation is that PGF2α has a direct positive effect on LH, despite the rapid clearance of PGF2α from the circulatory system [42]. In this regard, heifers treated with FM had lower mean PGFM and LH concentrations during various components of PGFM and LH pulses (Table 1). In addition, the greater frequency of the peak of PGFM pulse at the peak of an LH fluctuation than at Nadir 2 in the preluteolytic subgroup was consistent with a positive effect of PGF2α on LH. This synchrony between peaks of PGFM and LH was a novel observation, but was consistent with the reports that LH increases during estradiol-induced [15], spontaneous [18], and simulated [16] PGFM pulses. However, a stimulatory effect of LH on COX 2 and subsequently on PGF2α secretion through the LH receptors in the uterus has also been reported [43,44]. More study will be needed on the positive and negative effects among episodes of PGF2α, P4, and LH.

Previous reports [9,10] indicated on a temporal basis that LH stimulated P4 production by the CL during the preluteolytic period and during early luteolysis but not during late luteolysis. It was postulated that the positive effect of LH on P4 was balanced periodically against the negative effect of the PGFM pulses on P4 production during preluteolysis and early luteolysis. Pulses of LH accounted, at least in part, for the P4 rebound after the suppression at the peak of a PGFM pulse. Unlike the preluteolytic period, the P4 rebound during the luteolytic period did not reach the concentration present before the PGFM pulse [9]. The greater positive effect of LH pulses on P4 production during the preluteolytic period than during the luteolytic period [9] was confirmed for each group in the present study, as indicated by the greater frequency of

synchronized peaks of P4 fluctuations and LH pulses in the preluteolytic subgroup (75%) than in the luteolytic subgroup (38%) and the increase in P4 concentration at the peak of an LH pulse in each group in the preluteolytic subgroup (Fig. 7) but not in the luteolytic subgroup. The similarity between the vehicle and FM groups in the synchrony between LH pulses and P4 fluctuations in the preluteolytic subgroup indicated that inhibition of PGF2α pulses did not affect the LH/P4 synchrony. During in vitro culture, LH stimulates P4 production by small luteal cells, but not by large luteal cells [45]. The PGF2α inhibits parts of the steroidogenic pathway, resulting in a negative effect on the P4 production within large luteal cells through the protein kinase C second messenger system [46]. Treatment with PGF2α also induces loss of LH receptors and/or interferes with a deprivation of the enzymes/substrates related to the P4 production system within luteal cells [47]. The diminished stimulation of P4 production by LH during the luteolytic period may be attributable to the loss of LH receptors and viability of the regressing luteal cells. Studies are needed on mechanisms that apparently reduced LH stimulation of P4 production during luteolysis.

In conclusion, PGF2α secretion during the preluteolytic and luteolytic periods, based on the corresponding subgroups, was inhibited by an FM dose (2.5 mg/kg) given every 8 h for three treatments, compared to a dose of 0 mg (vehicle group). In heifers that were in the preluteolytic period at the first FM treatment (Hour 0), the prominence of PGFM pulses was diminished and a decrease in P4 from Hour 0 was delayed. In addition, there was a tendency for a greater P4 concentration at Hour 24, length of the luteolytic period, and fewer heifers with the beginning of luteolysis during the day of FM treatment. Based on PGFM concentration, FM treatment was more effective during preluteolysis than during luteolysis. Greater prominence of sequential PGFM pulses during luteolysis was related to a shorter time for completion of luteolysis. During preluteolysis in both the vehicle and FM groups, the peak of a PGFM pulse occurred more frequently at the same hour as the peak of an LH fluctuation than at the ending nadir of an LH fluctuation and was compatible with an association between the peak of a PGF2α pulse and an LH fluctuation.

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CAPÍTULO 3- STUDY 2

ESTUDO ENVIADO PARA PUBLICAÇÃO

Effect of dose of estradiol-17β on prominence of an induced 13,14-dihydro-15-keto-PGF2α (PGFM) pulse and relationship of prominence to progesterone, LH, and luteal blood flow in heifers

G. Pugliesi[a,b,c], M.A. Beg[b], G.R. Carvalho[c], O.J. Ginther[a,b]

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Effect of dose of estradiol-17β on prominence of an induced 13,14-dihydro-15-keto-PGF2α (PGFM) pulse and relationship of prominence to progesterone, LH, and luteal blood flow in heifers

(Efeito da dose de estradiol-17β na proeminência do pulso de um metabólito da PGF2α - PGFM e a relação entre a proeminência com a progesterona, LH e fluxo sanguíneo luteal em novilhas)

G. Pugliesi[a,b,c], M.A. Beg[b], G.R. Carvalho[c], O.J. Ginther[a,b]

[a]Eutheria Foundation, Cross Plains, Wisconsin 53528, Estados Unidos da América

[b]Department of Pathobiological Sciences, University of Wisconsin, Madison, Wisconsin 53706, Estados Unidos da América

[c]Departamento de Zootecnia, Universidade Federal de Viçosa,Viçosa, Minas Gerais, Brasil

RESUMO

Várias doses de estradiol-17β (E2) foram utilizadas em novilhas para induzirem um pulso de 13,14-dihydro-15-keto-PGF2α (PGFM). O efeito da concentração de E2 na proeminência dos pulsos de PGFM e sua relação com as concentrações de progesterona (P4), hormônio luteinizante (LH), e fluxo sanguíneo luteal foram estudas. Uma única dose de 0 (veículo), 0,01, 0,05, ou 0,1 mg de E2 foi administrado (n=6/grupo) 14 dias após a ovulação. Amostras de sangue foram colhidas e o fluxo sanguíneo luteal estimado de hora em hora por 10 horas logo após o tratamento. A dose de 0,05-mg resultou em aumento intermediário e a dose de 0,1-mg promoveu aumento mais pronunciado no pulso de PGFM, comparado com as doses de 0,0-mg e 0,01-mg. Os pulsos de PGFM foram subdivididos em três diferentes categorias de proeminência (< 50, 50–150, e > 150 pg/mL no pico do pulso). Na categoria de 50–150, a concentração de P4 ($P < 0,05$) entre –2 h e 0 h (0 h = pico do pulso de PGFM). Na categoria >150, a concentração de P4 reduziu entre –1 h e 0 h, a de LH

aumentou (P < 0,05) após uma hora do pico de PGFM, e o fluxo sanguíneo luteal aparentemente reduziu (P < 0,05) 2 h após o pico de PGFM. Os novos resultados suportaram as seguintes hipóteses: 1) aumento nas concentrações de E2 eleva a proeminência do pulso de PGFM, e 2) a maior proeminência do pulso de PGFM é associado com maior depressão de P4 no pico do pulso de PGFM. Adicionalmente, a dimensão do efeito da PGF2α no aumento das concentrações de LH e nas mudanças do fluxo sanguíneo luteal dentro das horas de um pulso de PGFM foi positivamente relacionada com a proeminência deste pulso de PGFM.
Palavras-chaves: Estradiol; LH; Luteólise; pulsos de PGFM; Progesterona

ABSTRACT

Various doses of estradiol-17β (E2) were used in heifers to induce a pulse of 13,14-dihydro-15-keto-PGF2α (PGFM). The effect of E2 concentration on the prominence of PGFM pulses and the relationship between prominence and intrapulse concentration of progesterone (P4), LH, and luteal blood flow were studied. A single dose of 0 (vehicle), 0.01, 0.05, or 0.1 mg of E2 was given (n=6/group) 14 days after ovulation. Blood samples were collected and luteal blood flow was evaluated hourly for 10 h after the treatment. The 0.05-mg dose increased and the 0.1 mg further increased the prominence of the induced PGFM pulse, compared to the 0.0-mg dose and the 0.01-mg dose. The PGFM pulses were subdivided into three different prominence categories (< 50, 50–150, and > 150 pg/mL at the peak). In the 50–150 category, P4 concentration increased (P < 0.05) between –2 h and 0 h (0 h = peak of PGFM pulse). In the >150 category, P4 decreased (P < 0.05) between –1 h and 0 h, LH increased (P < 0.05) at 1 h, and luteal blood flow apparently decreased (P < 0.05) at 2 h of the PGFM pulse. The novel results supported the following hypotheses: 1) an increase in E2 concentration increases the prominence of a PGFM pulse, and 2) greater prominence of a PGFM pulse is associated with a greater transient intrapulse depression of P4 at the peak of the PGFM pulse. In addition, the extent of the effect of PGF2α on the increase in LH and changes in blood flow within the hours of a PGFM pulse was related positively to the prominence of the PGFM pulse.
Keywords: Estradiol; Heifers; LH; Luteolysis; PGFM pulses; Progesterone

1. Introduction

In heifers, secretion of prostaglandin F2α (PGF2α) from the uterus induces luteolysis [1,2], and the onset of luteolysis begins 15, 16, or 17 days after ovulation [3]. Owing to rapid metabolism in the lungs and short half-life of PGF2α [4], the concentrations of the PGF2α metabolite 13,14-dihydro-15-keto-PGF2α (PGFM) often are used to represent the secretion of PGF2α. The half-life of PGFM is about 8 min, compared to < 1 min for PGF2α [4]. A sampling interval of 1 h has been recommended for detection of PGFM pulses in cattle [5,6]. Each pulse of PGFM is several hours in duration [7] and sequential pulses of PGF2α, as determined by PGFM concentrations, are needed to induce complete luteolysis in cattle [8], horses [9], and sheep [10].

The regulation of PGF2α secretion and subsequent timing of luteolysis in cattle involves expression of receptors for estradiol (E2), progesterone (P4), and oxytocin in the endometrium [11,12]. The concentration and day of exposure of uterus to E2, P4, and oxytocin are involved in the stimulation of PGF2α production [13–15]. Exposure of the uterus to P4 for 10 to 14 days after ovulation is required for an increase in the capacity of the endometrium for PGF2α secretion [11]. The E2 from developing ovarian follicles has been proposed to have a pivotal role in the induction of luteolysis in heifers [15–17] and sheep [18]. In this regard, luteolysis is delayed following destruction of ovarian follicles by electrocautery in sheep [19] and x-ray irradiation [16,20] or aspiration of follicle contents in heifers [15].

The PGFM pulses during luteolysis are more prominent than during preluteolysis [3,7] and are temporally associated with a greater concentration of E2 during early luteolysis than during preluteolysis [21]. A pharmacologic dose (1 or 3 mg) of E2 given intramuscularly or intravenously induces PGF2α secretion within 6 to 8 h [14,22]. A treatment of 0.1mg of E2 [23,24] given 14 days after ovulation stimulates a prominent pulse of PGFM. A single treatment of 0.1 mg of E2 results in a concentration of E2 [23] similar to reported [25] concentrations in the preovulatory E2 surge in heifers. Although the dose was considered physiological with reference to the preovulatory surge, the resulting concentration was greater than the concentration at the onset of luteolysis in

cattle [26–28]. Thus, the effect of treatment with a physiological dose of E2 on stimulation of PGF2α production is not known.

During the last PGFM pulse of the preluteolytic period, P4 decreases concomitantly with the increase in the ascending portion of the PGFM pulse. The P4 suppression at the PGFM peak is followed by an increase or rebound in P4 to a concentration that is similar to the concentration before the pulse [3,28]. A P4 rebound also occurs after the peak of an E2-induced PGFM pulse [23,24] and 1 or 2 h after the beginning of infusion of PGF2α to simulate a spontaneous PGFM pulse [29,30]. The negative effect of PGF2α on P4 concentration is balanced against a positive effect of LH pulses during the end of the preluteolytic period and early in luteolysis, based on temporal relationships [21,31]. The LH increase during an LH pulse contributes to the increase in P4 during a P4 fluctuation [32], including the P4 rebound that occurs after the P4 suppression at the peak of a PGFM pulse [21].

During spontaneous luteolysis, the percentage area of the corpus luteum (CL) with color-Doppler signals of blood flow increases during the ascending portion of a PGFM pulse, reaches maximum at the peak of the pulse, and decreases after two hours [2]. The luteal blood flow also increases in heifers concomitantly with the P4 rebound after the peak of an E2-induced PGFM pulse during the preluteolytic period [24] and during infusion of PGF2α to simulate a PGFM pulse [29].

The purpose of the present experiment was to evaluate the following hypotheses: 1) an increase in E2 concentration increases the prominence of a PGFM pulse, and 2) greater prominence of a PGFM pulse is associated with a greater transient intrapulse depression of P4 at the peak of the PGFM pulse. In addition, the relationships between prominence of the PGFM pulse and the intrapulse changes in LH and luteal blood flow were studied.

2. Materials and methods

2.1. Animals

Twenty-four dairy heifers (Holsteins) aged 18 to 24 mo and weighing 530 ± 18 kg (range: 490 to 580 kg) were used during June and July in the northern

temperate zone. Heifers were selected with docile temperament and no apparent abnormalities of the reproductive tract, as determined by ultrasound examinations [33]. If the heifer presented more than one CL or the single CL was considered undersized (< 3 cm^2) on 13 days after ovulation, the heifer was not used. The day of ovulation was determined by daily transrectal ultrasound examinations [33]. The heifers were kept under natural light in an open shelter and outdoor paddock and were maintained by *ad libitum* access to a mixture of alfalfa and grass hay, water, and trace-mineralized salt. Heifers remained healthy and in good body condition throughout the experiment.

2.2. Follicle ablations

Ultrasound-guided transvaginal ablation of ovarian follicles was performed on 13 days after ovulation to remove the follicle source of the circulating E2, with the expectation that follicle ablation would favor an increase in the synchrony of PGFM pulses after an E2 treatment; synchrony was not obtained when an E2 treatment was given without previous follicle ablation [23,24]. Follicle ablation was performed as described [34]. Briefly, a duplex B-mode (gray-scale) and pulsed-wave color Doppler ultrasound instrument (Aloka SSD 3500; Aloka America, Wallingford, CT) equipped with a 5.0-MHz sector and 7.5-MHz linear transducers was used. The area (cm^2) of the CL and diameter of follicles was obtained by transrectal ultrasonography. Follicle ablation was done during transvaginal scanning of the follicles and transrectal ovarian manipulation. A 17-gauge needle was connected to a vacuum pump and inserted through the transvaginal transducer and into each follicle ≥ 4 mm. The fluid contents were removed under vacuum suction (≈130 mm Hg). Caudal epidural anesthesia (2 mL of 2 % lidocaine hydrochloride) was used to minimize abdominal straining and to facilitate the ovarian manipulation. After each procedure, the ovaries were reexamined by transrectal ultrasonography to verify that all follicles ≥ 4 mm were ablated successfully.

2.3. Experimental design and treatments

Heifers were assigned randomly by replicate (n = 6) to one vehicle-treated and three E2-treated groups. Vehicle and E2 groups were treated at 7:00 a.m. 14 days after ovulation, when heifers were expected to be in preluteolysis [2,3]. Heifers in the vehicle group were given a single intramuscular treatment of vehicle (2 mL of sesame oil). The E2 doses were prepared in a sesame oil vehicle as described [23]. Heifers in the E2 groups were given the assigned dose of E2 in a single 2 mL intramuscular treatment. The three E2-treated groups involved doses of 0.01 mg, 0.05 mg, and 0.1 mg of E2. The E2 doses were selected to include the reported [23,35] doses that would result in physiological concentrations of E2 after a single E2 treatment. The hour of treatment was designated Hour 0.

A blood sample (10 mL) was taken each hour from Hour 0 to Hour 10 to study the hourly changes in concentrations of PGFM, P4, and LH and the number and location of pulses of PGFM and LH. The hourly blood samples were collected through an indwelling catheter that was inserted into a jugular vein and secured as described [36]. The catheter was inserted 15 h before collection of the first hourly sample. Heifers had free access to water and hay between collection of samples. In addition, blood samples were taken from the tail vein daily from 13 days after ovulation until the detection of regression of the CL (luteal area, ≤ 2 cm^2) or until 24 days after ovulation if regression of CL was not detected. The daily samples were used to determine the day of the end of luteolysis in each heifer. The end of luteolysis was defined as occurring at the hour of the sample when P4 decreased to < 1 ng/mL [2,27].

Percentage of area (cm^2) of the CL with color-Doppler signals of blood flow was determined as described [2] at each hour just after the blood-sample collection. Letter-coded vials of E2 doses for the four experimental groups were used so that the operator did not know the group assignment for each heifer. All scans were performed at a constant color-gain setting and a velocity setting of 6 cm/sec. The color Doppler-transducer was passed over the entire CL and the images were stored on film. After recording the images from all heifers, the film clips were evaluated without knowledge of groups or hour to estimate the percentage of luteal area with color-Doppler signals as described [37].

2.4 Identification of PGFM and LH pulses

In each individual heifer, a transient hormone increase and decrease (fluctuation) encompassing at least four values (PGFM) or three values (LH, [31]) including nadirs with a progressive increase on the ascending portion and progressive decrease on the descending portion were evaluated. The CV of hormone concentrations at the hours of the fluctuation and the intra-assay CV were used to distinguish fluctuations from pulses of PGFM and LH as described [2]. When the CV of the values composing a fluctuation of PGFM and LH was at least three times greater than the mean intra-assay CV, the fluctuation was defined as a pulse. These definitions were based on reports that episodes of PGFM [5,6] and LH [6,31] at this stage of the estrous cycle can be detected by sampling blood at 1-h intervals. Pulses of PGFM and LH with a peak near the beginning or end of the hourly-sampling session were included in the CV analyses only if they were preceded or followed, respectively, by at least one lower value, but the lower value was not used to calculate nadir concentrations. Nadirs 1 and 2 were defined as the nadirs at the beginning and end of a PGFM or LH pulse.

The CV-identified PGFM pulses for the vehicle-treated and each of the three E2-treated groups were subdivided into three different categories to study the effect of the prominence of the PGFM pulse on the intrapulse changes in P4, LH, and luteal blood flow. The prominence categories were based on the concentration at the peak of PGFM pulses of < 50, 50–150, and > 150 pg/mL. The categories of prominence of PGFM pulses were developed so that an adequate number of pulses (n = 8–10) were available for each category and without knowledge of the P4, LH, and blood-flow results.

2.5 End points

Blood samples from heifers during a luteolytic period of a previous study (unpublished) were used to compare the E2 concentration at the peak of spontaneous versus E2-induced PGFM pulses. For the E2 assay, the blood samples (n = 4 heifers) for the spontaneous PGFM pulses were randomized with those of the present experiment. The E2 concentrations were compared

among groups for Hour 1, at the peak of the PGFM pulse in each heifer in the E2 groups, and at the peak of the spontaneous PGFM pulses.

The four experimental groups (vehicle, 0.01-mg E2, 0.05-mg E2, and 0.1-mg E2) were compared for the mean over all hours of the 10-h session and for the characteristics of the PGFM pulse with the greatest peak during a 10-h session. The pulse concentrations were for Nadir 1, peak, Nadir 2 and the amplitude (difference between peak and Nadir 1). Area under the pulse curve and intervals from treatment (Hour 0) to Nadir 1 and to the peak and from Nadir 1 to 2 were also considered. The concentration of PGFM for –2 h to 2 h (0 h = pulse peak) of the PGFM pulse with the greatest peak per 10-h session was compared among the four groups. The maximal LH concentration and the characteristics of the LH pulses during a 10-h session also were compared among groups. The LH pulse concentrations were for Nadir 1, peak, Nadir 2, and amplitude. The interval from Nadir 1 to 2 of the LH pulse also was considered.

The PGFM concentration of pulses for the three prominence categories (peaks of < 50, 50–150, and > 150 pg/mL) were compared for –2 h to 2 h (0 h = pulse peak). The hour of the peak of a PGFM pulse was used for centralization and comparison among the three prominence categories of PGFM pulses of the intrapulse changes (–2 h to 2 h) in concentration of PGFM, P4, and LH and for the percentage of luteal area with color-Doppler signals of blood flow. In addition, the maximal LH concentration within the hours of a PGFM pulse and the concentration at the peak of an LH pulse that occurred within the PGFM pulse were compared among the three prominence categories of PGFM pulses. The peaks of LH pulses were subdivided into those that occurred before (at –2 h or –1 h) or after (at 1 h or 2 h) the peak of a PGFM pulse. The peak of an LH pulse that occurred at the same hour as the peak of a PGFM pulse (0 h) was not included in the before and after partitioning.

2.6 Hormone assays

The blood samples were immediately placed in ice water for 10 min before centrifuging (2000 X *g* for 10 min). The plasma was decanted and stored (–20 ºC) until assay. The plasma samples were assayed for PGFM by an ELISA that

was developed and validated in our laboratory for use with bovine plasma [7]. Plasma P4 concentrations were assayed with a solid-phase RIA kit containing antibody-coated tubes and I^{125}-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA). The procedure has been validated and described [2] for bovine plasma in our laboratory. The plasma concentrations of LH were determined by RIA as described [38] and modified [39] for bovine plasma in our laboratory. Plasma E2 concentrations were assayed as described and validated in our laboratory for bovine plasma [40], using a commercial RIA kit (Double Antibody Estradiol: Diagnostic Products Corporation). The intra- and interassay CV and sensitivity for PGFM, respectively, were 8.2%, 16.0%, and 17.9 pg/mL. The intra-assay CV and sensitivity for P4, LH, and E2, respectively, were 8.6% and 0.001 ng/mL, 16.1% and 0.04 ng/mL, and 2.9% and 0.07 pg/mL.

2.7 Statistical analyses

Outlying observations greater than three interquartile ranges from the mean were not used in the statistical analyses (PROC UNIVARIATE; SAS online Doc 9.1.3 SAS Institute Inc., Cary, NC, USA). Data were examined for normality using Shapiro-Wilk test. Data that were not normally distributed were transformed to natural logarithms or ranks. The differences for each hormone were analyzed for the main effect of group and hour and for the interaction. The SAS PROC MIXED procedure (Version 9.2; SAS Institute) was used with a REPEATED statement to account for autocorrelation between sequential measurements. The LSD test was used when comparisons between hours were made within a group or among more than two means. Hormone concentrations during CV-identified PGFM pulses were compared among the prominence categories of PGFM pulses in an hour-by-category analysis using the peak (0 h) of a PGFM pulse as a centralized reference point for another hormone and for the percentage of blood flow. The LSD test was used to locate differences among categories and among hours within each category. Discrete data for characteristics and intervals for PGFM pulses were analyzed for differences among groups by the LSD test. A Fischer's exact test was used for comparison of the frequency of occurrence of PGFM pulses among groups. Student's

unpaired *t*-tests were used to locate differences between the concentrations of an LH peak within each category of PGFM pulses. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $P > 0.05$ to $P \leq 0.1$ indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise indicated.

3. Results

Based on the daily samples, the interval from pretreatment ovulation to end of luteolysis was not different ($P > 0.1$) between vehicle and 0.01-mg E2 groups and between 0.05-mg E2 and 0.1-mg E2 groups. The interval from pretreatment ovulation to end of luteolysis was longer in the combined vehicle and 0.01-mg E2 groups than in the combined 0.05-mg E2 and 0.1-mg E2 groups (19.1 ± 0.4 d vs. 17.5 ± 0.4 d, $P < 0.005$).

The probabilities for a group effect, hour effect, and a group-by-hour interaction for the factorial analyses are given in the figures and the probabilities for differences in discrete end points are given in the table or text. There was a difference ($P < 0.0001$) among groups in the mean E2 concentration at Hour 1 as follows: vehicle (0.3 ± 0.1 pg/mL^{d}), 0.01-mg E2 (1.8 ± 0.3 pg/mL^{c}), 0.05-mg E2 (7.7 ± 1.2 pg/mL^{b}), and 0.1-mg E2 (22.5 ± 4.8 pg/mL^{a}); means with a different superscript are different ($P < 0.05$). At the hour of the peak of a PGFM pulse, the E2 concentration in spontaneous PGFM pulses and among the E2-treated groups were as follows: spontaneous (1.7 ± 0.3 pg/mL^{c}), 0.01-mg E2 (1.1 ± 0.2 pg/mL^{c}), 0.05-mg E2 (2.8 ± 0.3 pg/mL^{b}), and 0.1-mg E2 (3.8 ± 0.3 pg/mL^{a}); means with a different superscript are different ($P < 0.05$).

Mean concentrations of PGFM for the 10-h sessions of hourly sampling showed main effects of group and hour and an interaction of group by hour (Fig. 1). The group effect represented greater PGFM concentrations averaged over the 10 h in the 0.1-mg E2 group than in the vehicle, 0.01-mg E2, and 0.05-mg E2 groups (Table 1). The interaction reflected an increase ($P < 0.05$) in PGFM concentrations between Hours 4 and 5 in the 0.05-mg E2 group and an increase ($P < 0.05$) between Hours 2 and 3 in the 0.1-mg E2 group, compared to no increase in vehicle or 0.01-mg groups. Concentrations were greater ($P <$

0.05) in the 0.05-mg E2 and 0.1-mg E2 groups than in the vehicle and 0.01-mg E2 groups at Hours 5, 6, 7 and 8.

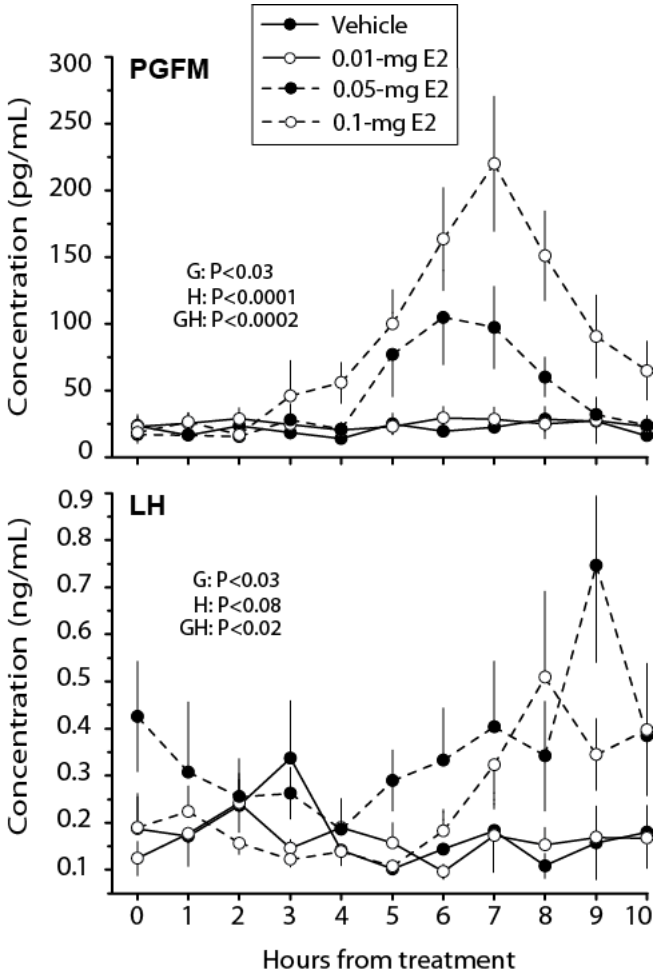

Fig. 1. Mean ± SEM for concentrations of PGFM and LH from treatment (Hour 0) in a vehicle group and in three groups treated with doses of estradiol-17β of 0.01 mg, 0.05 mg or 0.1 mg on 14 days after ovulation (n = 6 heifers/group). Main effects of group (G) and hour (H) and interaction (GH) that were significant or approached significance are shown.

For the hourly concentrations of P4 (range of means/session, 6.2–10.0 ng/mL), there were no differences during the 10-h sessions within each group and in the factorial analysis among the four groups (data not shown). The mean hourly concentrations of LH showed main effects of group, an effect of hour that approached significance, and an interaction of group by hour (Fig. 1). The group effect represented greater ($P < 0.05$) LH concentrations averaged over the 10 h in the 0.05-mg E2 and 0.1-mg E2 groups than in the vehicle and 0.01-mg E2 groups (Table 2). The interaction reflected a first increase ($P < 0.05$) in LH concentration between Hours 4 and 9 in the 0.05-mg E2 group and a first

increase ($P < 0.05$) between Hours 5 and 7 in the 0.1-mg E2 group, compared to no increase in the vehicle and 0.01-mg E2 groups. There was greater maximal LH concentration during the 10-h session in the 0.05-mg E2 and 0.1-mg E2 groups than in the vehicle and 0.01-mg E2 groups.

Table 1. Mean ± SEM characteristics of the PGFM pulse with the greatest peak/heifer and length of intervals between events in a vehicle group and in three E2-treated groups with doses of estradiol-17β of 0.01 mg, 0.05 mg, or 0.1 mg 14 days after ovulation.

End point	Group				Probability
	Vehicle	0.01-mg E2	0.05-mg E2	0.1-mg E2	
No. of heifers	6	6	6	6	
No. of heifers with a PGFM pulse	3[b]	6[a]	6[a]	6[a]	P < 0.04
Mean PGFM during 10-h sampling session (pg/mL)*	21.3 ± 3.9[b]	25.3 ± 7.7[b]	43.8 ± 9.9[b]	86.7 ± 17.1[a]	P < 0.001
PGFM pulse concentration (pg/mL)					
Nadir 1[#]	22.9 ± 4.7	14.9 ± 5.5	18.0 ± 4.9	33.0 ± 11.8	NS
Peak	53.1 ± 6.8[b]	44.5 ± 15.0[b]	124.0 ± 34.8[b]	231.8 ± 43.5[a]	P < 0.003
Nadir 2[#]	5.8 ± 1.7	15.4 ± 4.4	19.5 ± 8.5	22.2 ± 12.6	NS
Amplitude[†]	30.2 ± 6.9[b]	25.4 ± 13.0[b]	106.0 ± 33.3[ab]	198.7 ± 44.0[a]	P < 0.008
Area under pulse curve (pg/mL x h)	83.5 ± 3.7[b]	56.9 ± 26.4[b]	286.9 ± 105.5[ab]	635.9 ± 165.0[a]	P < 0.007
Intervals (h)					
Hour 0 to Nadir 1	4.7 ± 1.9	2.3 ± 0.6	4.2 ± 0.5	3.8 ± 0.5	NS
Hour 0 to peak	6.0 ± 2.0[ab]	3.5 ± 1.2[b]	6.3 ± 0.4[a]	7.3 ± 0.2[a]	P < 0.03
Nadir 1 to Nadir 2	3.0 ± 0.0[b]	3.0 ± 0.0[b]	4.8 ± 0.8[a]	5.0 ± 0.0[a]	P < 0.04

* Hourly blood sampling was done from Hours 0 to 10 (Hour 0 = treatment) in heifers in preluteolysis.
[#] Nadirs 1 and 2 are the nadirs at the beginning and the end of a PGFM pulse, respectively.
[†] Amplitude is the difference in concentration between Nadir 1 and peak.
[ab] Means within a row without a common superscript are different (P < 0.05).

The CV-identified pulses of PGFM were detected in three of six heifers in the vehicle group (total of four pulses) and in six of six heifers in each of the 0.01-mg E2 (eight pulses), 0.05-mg E2 (seven pulses), and 0.1-mg E2 groups (eight pulses). Only the pulse with the greatest peak was used for the comparison among groups. The interval from treatment to the Nadir 1 of the PGFM pulse with the greatest peak did not differ among groups, but the interval

from treatment to the peak was longer ($P < 0.03$) for heifers in the 0.05-mg E2 and 0.1-mg E2 groups than in the 0.01-mg E2 group (Table 1). There was apparent synchrony of the peak of the PGFM pulses at a mean of Hour 6.3 in the 0.05-mg E2 group (Hours of each peak: 5, 6, 6, 6, 7, and 8) and at a mean of Hour 7.3 in the 0.1-mg E2 group (Hours: 7, 7, 7, 7, 8, and 8); no difference between groups.

Table 2. Mean ± SEM characteristics of LH pulses and length of intervals between LH events in a vehicle group and in three E2-treated groups with doses of estradiol-17β of 0.01 mg, 0.05 mg, or 0.1 mg 14 days after ovulation.

End point	Group				Probability
	Vehicle	0.01-mg E2	0.05-mg E2	0.1-mg E2	
No. of heifers	6	6	6	6	
No. of LH pulses/10-h	1.8 ± 0.3	2.2 ± 0.4	2.3 ± 0.6	1.7 ± 0.2	NS
During 10-h sampling session (ng/mL)*					
Mean LH concentration	0.18 ± 0.02[b]	0.17 ± 0.02[b]	0.34 ± 0.04[a]	0.25 ± 0.03[a]	P < 0.03
Mean of maximum concentration	0.47 ± 0.1[b]	0.45 ± 0.04[b]	0.87 ± 0.11[a]	0.86 ± 0.08[a]	P<0.005
LH pulse concentration (ng/mL)					
Nadir 1[#]	0.09 ± 0.01	0.09 ± 0.01	0.13 ± 0.03	0.10 ± 0.01	NS
Peak	0.42 ± 0.07[b]	0.33 ± 0.03[b]	0.50 ± 0.05[ab]	0.58 ± 0.06[a]	P < 0.03
Nadir 2[#]	0.08 ± 0.01[b]	0.09 ± 0.01[b]	0.14 ± 0.04[ab]	0.16 ± 0.03[a]	P < 0.05
Amplitude[†]	0.32 ± 0.07[ab]	0.24 ± 0.03[b]	0.37 ± 0.04[ab]	0.47 ± 0.06[a]	P < 0.04
Interval from Nadir 1 to 2 (h)	3.7 ± 0.2	3.6 ± 0.3	3.0 ± 0.4	3.7 ± 0.4	NS

* Hourly blood sampling was done from Hours 0 to 10 (Hour 0 = treatment) in heifers in preluteolysis.
[#] Nadirs 1 and 2 are the nadirs at the beginning and the end of a LH pulse, respectively.
[†] Amplitude is the difference in concentration between Nadir 1 and peak.
[ab] Means within a row without a common superscript are different (P < 0.05).

Concentration at the peak of the PGFM pulse was greater for heifers in the 0.1-mg E2 group than in the other three groups (Table 1). The amplitude and area under the curve were greater in the 0.1-mg E2 group than in the vehicle and 0.01-mg E2 groups. The number of heifers with PGFM pulses with a peak > 150 pg/mL was greater ($P < 0.05$) in the 0.05-mg E2 group (3 of 6) and 0.1-mg E2 group (5 of 6) than in the vehicle group (0 of 6) and 0.01-mg E2 group (0 of 6). The concentrations at Nadir 1 and Nadir 2 did not differ among groups. The interval between Nadir 1 and Nadir 2 was longer for heifers in the 0.05-mg E2 and 0.1-mg E2 groups than in 0.01-mg E2 group.

A group effect and hour effect were detected for PGFM concentration from –2 h to 2 h of the PGFM pulse/heifer (Fig. 2). The group effect reflected the following PGFM concentration averaged over –2 h to 2 h (0 h = peak): vehicle (33.7 ± 4.9 pg/mL^{b}), 0.01-mg E2 (31.4 ± 5.5 pg/mL^{b}), 0.05-mg E2 (75.3 ± 12.8 pg/mL^{ab}), and 0.1-mg E2 (145.5 ± 17.5 pg/mL^{a}); means without a common superscript are different ($P < 0.05$). The hour effect represented a greater concentration at the peak of pulses, as expected from centralizing PGFM concentrations to the peak.

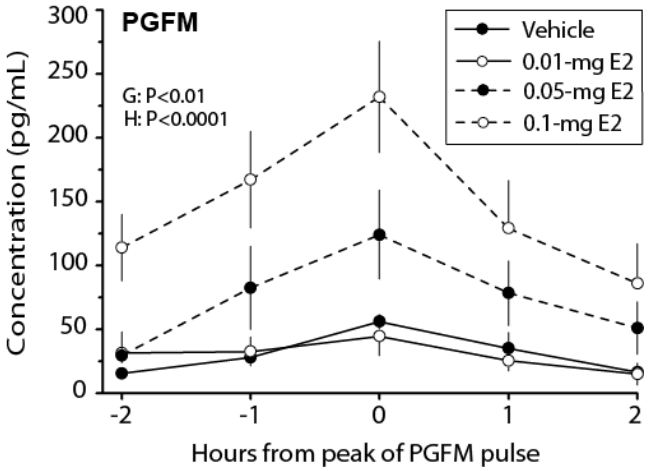

Fig. 2. Mean ± SEM intrapulse concentration of PGFM centralized to the peak of the PGFM pulse with the greatest peak/heifer in a vehicle group and in three groups treated with doses of estradiol-17β of 0.01 mg, 0.05 mg, or 0.1 mg on 14 days after ovulation (n = 6 heifers/group). Main effects of group (G) and hour (H) that were significant are shown.

The number of LH pulses/10-h session did not differ among groups (Table 2). The LH concentration at the peak and Nadir 2 was greater for heifers in the 0.1-mg E2 group than in the vehicle and 0.01-mg E2 groups. The amplitude was greater in the 0.1-mg E2 group than in the 0.01-mg E2 group. The LH concentration at Nadir 1 and the interval between Nadir 1 and Nadir 2 did not differ among the groups.

The PGFM, P4, and LH concentrations and luteal blood flow within PGFM pulses for the three prominence categories of < 50, 50–150, or > 150 pg/mL at the peak are shown (Fig. 3). The PGFM concentration showed a category and an hour effect but no interaction. The category effect represented the concentrations averaged over –2 h to 2 h in the following PGFM-pulse categories: <50 (21.7 ± 1.9 pg/mL^{c}), 50–150 (45.1 ± 4.2 pg/mL^{b}), and >150

(147.7 ± 13.7 pg/mL[a]); means with a different superscript are different (P < 0.05).

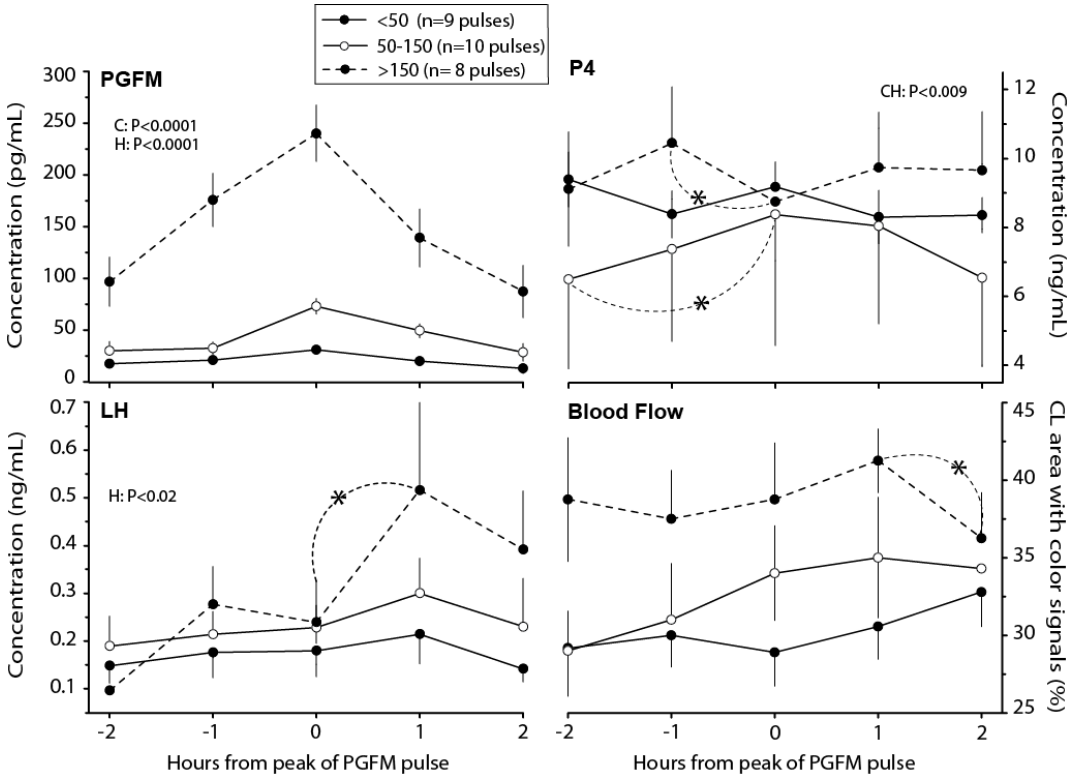

Fig. 3. Mean ± SEM intrapulse concentration of PGFM, P4, and LH and the percentage of luteal area with signals of blood flow centralized to the peak of PGFM pulses in three categories of prominence of PGFM pulses (<50, 50–150, and >150) on 14 days after ovulation. The categories were based on the concentration at the peak of PGFM pulses of < 50, 50–150 and > 150 pg/mL. Main effects of category (C) and hour (H) and their interaction (CH) that were significant are shown. A decrease or increase (P < 0.05) between two hours in P4 or LH concentrations or in the percentage of luteal area with signals of blood flow are indicated by an asterisk between the two hours.

For P4 concentration centralized to the PGFM peak (0 h), only an interaction of category by hour was observed. The interaction represented in part a difference in concentrations among hours for the 50–150 category (P < 0.05) and the >150 category (P < 0.05) but not for the <50 category (P > 0.1). An increase (P < 0.05) in P4 concentration occurred between –2 h and 0 h in the 50–150 category and a decrease (P < 0.05) between –1 h and 0 h in the >150 category.

For LH concentration within each PGFM prominence category, only an hour effect was observed. Although the interaction was not significant, a difference (P < 0.009) among hours was detected only for the >150 category and involved an increase (P < 0.05) between 0 h and 1 h of the PGFM pulse.

The LH concentration was greater ($P < 0.05$) in the >150 category than the <50 category at 1 h after the peak of the PGFM pulse. The peak of a CV-identified LH pulse and the maximal LH concentration during the 5 h between –2 h and 2 h of the PGFM pulses were greater in the >150 prominence category of PGFM pulses than in the other two categories (Table 3). The number of CV-identified LH pulses that reached a peak between –2 h and 2 h of the PGFM pulse was not different ($P > 0.1$) among categories. The number of LH pulses that occurred before or after the peak of a PGFM pulse did not differ ($P > 0.1$) among categories or within each category of PGFM pulses. The concentration at the peak of an LH pulse that occurred at –2 h or –1 h of the PGFM pulse was not different among the PGFM pulse categories. The LH peak that occurred at 1 h or 2 h of the PGFM pulse was greater in the >150 category than in the <50 and 50–150 categories. The peak concentration of LH pulses that occurred before the peak (at –2 h or –1 h) vs. after the peak (at 2 h or 1 h) of the PGFM pulse were not different in the <50 and 50–150 prominence categories of PGFM pulses, but the peak of an LH pulse that occurred after the peak of the PGFM pulse in the >150 category was greater ($P < 0.002$) than the peak of LH that occurred before the PGFM peak.

Table 3. Mean ± SEM concentration of LH pulses between –2 h and 2 h of the PGFM pulses (0 h = peak of PGFM pulse) in three prominence categories of PGFM pulses 14 days after ovulation.

LH concentration (ng/mL)	Prominence categories for PGFM pulses*			
	<50	50–150	>150	Probability
Maximal LH#	0.34 ± 0.06[b]	0.49 ± 0.09[b]	0.76 ± 0.08[a]	P < 0.004
LH pulse†				
LH peak within the PGFM pulse	0.40 ± 0.05[b]	0.48 ± 0.09[b]	0.72 ± 0.09[a]	P < 0.03
LH peak before PGFM peak	0.36 ± 0.05	0.52 ± 0.20	0.39 ± 0.04[x]	NS
LH peak after PGFM peak	0.38 ± 0.07[b]	0.39 ± 0.08[b]	0.84 ± 0.10[a,y]	P < 0.003

* The prominence categories were based on the concentration at the peak of PGFM pulses of < 50, 50–150 and > 150 pg/mL.
# Maximal LH concentration was considered as the LH concentration at the hour during the 5 h of the PGFM pulse that had the highest LH concentration.
† The LH concentration was considered only at the peak of the CV-identified LH pulses.
‡ The LH peak that occurred at –2 h and –1 h (before) and 1 h and 2 h (after) of peak of a PGFM pulse. The peak of an LH pulse that occurred at the same hour of the peak of a PGFM pulse (0 h) was not included.
[ab] Means within a row with a different superscript are different (P < 0.05).
[xy] Means for the LH pulse within the >150 category with a different superscript are different (P < 0.05).

A main effect of category or hour or an interaction were not detected for the percentage of luteal area with color-Doppler signals of blood flow, and there were no changes for any category when analyzed separately. However, blood flow in the >150 category of PGFM pulses decreased ($P < 0.05$) between 1 h and 2 h, based on a paired *t*-test.

4. Discussion

The present study evaluated the effect of doses of E2 on prominence of induced PGFM pulses during the preluteolytic period and the relationships between the prominence of a PGFM pulse and the intrapulse changes in LH, P4, and luteal blood flow. The heifers were in preluteolysis on the day of the experiment (14 days after ovulation) as indicated by the P4 concentrations. In addition, P4 did not decrease to below 1 ng/mL (beginning of postluteolysis [3]) until ≥ 18 days after ovulation in any of the 12 heifers in the vehicle and 0.01-mg E2 groups. In this regard, the reported length of the luteolytic period based on hourly sampling is 24 h [3]. Only one of the 12 heifers in the 0.05 and 0.1-mg groups reached the beginning of luteolysis by 15 days, and in the one heifer an apparent P4 decrease in the hourly samples at 14 days occurred after the induced PGFM pulse.

There was an apparent synchrony in the interval from Hour 0 to the peak of the induced PGFM pulse with the 0.05 mg and 0.1 mg doses of E2, as indicated by pulse peaks at Hours 5 to 8. The follicles were ablated before Hour 0 and ablation was expected to reduce the endogenous E2 concentrations as reported [35] and thereby increase the synchrony of the PGFM pulses. In previous studies [6,23,24] with 0.1 mg dose of E2 but without follicle ablation, the pulses did not appear to be synchronized; the PGFM peak occurred at Hours 2, 3, 4, 5, 6, or 7 in six heifers. However, it is premature to conclude that follicle ablation increased the synchrony of E2-induced PGFM pulses, owing to the absence of a group without ablation of follicles.

The single treatment with E2 doses (0.05 mg and 0.1 mg) that induced a PGFM pulse resulted in circulating E2 concentrations at Hour 1 (1 h after treatment) that seemed similar and greater, respectively, to the reported

concentrations [25] at the peak of a preovulatory E2 surge. Although the 0.05-mg dose can be considered physiological with reference to the preovulatory surge, the resulting concentrations were greater than expected for the day of treatment (14 days after ovulation). Concentrations at this time have been reported as < 1pg/mL [7]. At the hour of the peak of the induced PGFM pulses, E2 concentrations had decreased 64 to 73%. The E2 concentration was about twice as great as the E2 concentration at the peak of the spontaneous PGFM pulses and the reported concentrations [28] at the hour of transition between preluteolysis and luteolysis. However, the E2 concentrations at the peak of the second PGFM pulse during spontaneous luteolysis (2.9 ± 0.3, n = 6) in an ongoing study (unpublished) seems similar to the E2 concentrations at the peak of the induced pulses (2.8 ± 0.3 and 3.8 ± 0.3 pg/mL for the 0.05 and 0.1 mg doses, respectively).

Hypothesis 1 that increasing E2 concentration increases the prominence of PGFM pulses was supported. This was indicated by the positive association between the increase in the dose of E2 and the increase in PGFM concentration during the 10-h sessions and in the prominence of induced PGFM pulses. The lowest E2 dose (0.01 mg) was no more effective for inducing an increase in PGFM concentration than the 0 dose in the vehicle group. The PGFM concentration during a PGFM pulse and the characteristics of the pulses were not greater in the 0.05-mg E2 group than in the vehicle and 0.01-mg E2 groups. However, a partial effect on PGFM concentration in the 0.05-mg E2 group was indicated by the greater PGFM concentration at Hours 5 to 8 than in the vehicle and 0.01-mg E2 groups and by an increase in number of heifers with a PGFM pulse in the most prominent category (> 150 pg/mL at the peak). In addition, the interval between Nadir 1 and Nadir 2 in the 0.05-mg E2 was longer than in the vehicle and 0.01-mg E2 groups. A greater effect of E2 on PGFM in the 0.1-mg E2 group than in the vehicle and 0.01-mg E2 groups was indicated by the greater PGFM concentration averaged over the 10-h session and the greater concentrations at the peak, amplitude, area under curve, and the overall mean of the PGFM pulses. In addition, the 0.1-mg E2 dose stimulated a greater peak in the induced PGFM pulses than the 0.05-mg E2 dose. The PGFM concentrations at the peak of a PGFM pulse that was induced by the 0.1-mg E2 dose were similar to the previously reported peak concentrations for this dose

[6,23,24]. The greater E2-induced PGF2α production in the 0.05-mg and 0.1-mg E2 groups than in the other groups accounts for the shorter interval from pretreatment ovulation to end of luteolysis in these groups.

The increased prominence of PGFM pulses from the increased doses of E2 indicated that E2 has a direct role in the production of PGF2α, agreeing with the reports [11,41] that E2 is involved in PGF2α secretion. A direct effect of E2 on the enzymes and substrates involved in the PGF2α production has been suggested [11,42]. Furthermore, E2 is involved in the mRNA expression for oxytocin receptors in the endometrial cells, which binds the oxytocin molecules and allows stimulation of PGF2α biosynthesis [11,27,44]. Thus, the increase in the prominence of PGFM pulses reported during luteolysis [3,4] can be attributed to be an amplification of PGF2α synthesis by the increase in E2 concentration. In addition, the decrease in P4 during spontaneous luteolysis amplifies the E2 stimulation on expression of oxytocin receptors, and increases the pulsatile secretion of oxytocin from the neurohypophysis [11,45]. It is not known whether a decrease in P4 alone has a stimulatory effect on PGF2α secretion. The present study demonstrated, however, that an increase in E2 stimulates increased PGF2α secretion without an associated decrease in P4.

Hypothesis 2 that greater prominence of a PGFM pulse is associated with greater intrapulse depression of P4 concentration at the peak of the PGFM pulse was supported. This was indicated by a decrease in P4 concentration between −1 h and 0 h of the PGFM pulse only for pulses with > 150 pg/mL at the pulse peak (>150 prominence category), but not for the <50 and 50–150 categories. This is the first report on the positive relationship between the prominence of a PGFM pulse and the extent of the transient depression in P4.The P4 depression at the peak of an E2-induced PGFM pulse has been reported for the dose of 0.1 mg E2 [23,24]. In this regard, PGF2α reduces the luteal P4 production by inhibiting the constitutive steroidogenic pathway through the protein kinase C second messenger system within large luteal cells [46].

For the PGFM pulses in the intermediate-prominence category (peak, 50–150 pg/mL), P4 concentration increased unexpectedly during the ascending portion of the PGFM pulse, contrasting with the P4 decrease during the ascending portion of PGFM pulses in the most prominent category (>150 pg/mL). The negative effect of prominent pulses and the apparent positive effect

of nonprominent PGF2α pulses on P4 production suggest that the effect of PGF2α on P4 is dependent on the concentrations of PGF2α. However, the apparent stimulation of P4 by an intermediate PGF2α concentration on luteal P4 production was not part of Hypothesis 2 and requires confirmation and further study.

An LH increase during the hours of a PGFM pulse occurred only in the PGFM pulses with a peak > 150 pg/mL, similar to the P4 suppression in the >150 prominence category. The greater LH response with the prominent pulses of PGFM has not been reported previously, and was indicated in the present study by the greater maximal LH concentration and the greater peak of an LH pulse during a PGFM pulse in the >150 prominence category than in the <50 and 50–150 categories. An LH increase in the >150 category was also indicated by the apparent increase in LH concentration between 0 h and 1 h of a PGFM pulse and the greater peak of an LH pulse after than before the peak of the PGFM pulse. However, the increase between 0 h and 1 h is considered with reservation, owing to the absence of an interaction in the factorial analysis. The increase in LH after the peak of a PGFM pulse is consistent with the increase in LH concentration after the increase in PGFM concentrations during the10-h session in the 0.05 and 0.1-mg E2 groups. The association between a prominent PGFM pulse and the increase in LH concentration occurred during the time of the P4 rebound after the P4 suppression at the PGFM peak. This is consistent with an increase in LH concentration during the reported progesterone rebound associated with a spontaneous PGFM pulse during preluteolysis [6,20], during a simulated PGFM pulse by PGF2α infusion [30], and during induction of a PGFM pulse by the 0.1-mg dose of E2 [24]. The LH increase may be, at least in part, a response to the decrease in P4 concentration at the peak of PGFM pulse through the removal of the inhibitory action of P4 on LH release from hypophyseal cells [47].

Changes in luteal blood flow was observed only during the PGFM pulses with a peak >150 pg/mL, as for the suppression in P4 and increase in LH. A blood-flow change was indicated by the decrease in the percentage of luteal area with color-Doppler signals of blood flow between 1 h and 2 h after the peak of the PGFM pulse in the >150 category. However, this effect was detected by a paired *t*-test and not by the factorial analysis. An alteration in luteal blood flow is

compatible with previous reports that an increase in the luteal blood flow followed by a decrease after the peak of the PGFM pulse is temporally associated with a spontaneous PGFM pulse [2,48], an E2-induced PGFM pulse [24], and a simulated PGFM pulse [30]. Pharmacological treatment with a PGF2α analogue on days 10–12 after ovulation induces luteolysis and increases peripheral blood flow of the CL 30 min after the PGF2α treatment [48–50]. Several reports [50–53] have indicated that the alteration in blood flow in the CL during the beginning of luteolysis is involved with the expression of the vasoactive substances endothelial nitric oxide synthase, endothelin, and angiotensin II.

In conclusion, the prominence of E2-induced PGFM pulses increased with increasing E2 doses, indicating that E2 has a novel dose-dependent role in up-regulation of the endometrial mechanism involved in PGF2α synthesis that leads to the luteolytic process. The 0.01-mg E2 dose was ineffective in increasing PGFM concentration and in inducing a prominent PGFM pulse, whereas 0.05 mg was partially effective and 0.1 mg was more effective. A PGFM pulse with a peak < 50 pg/mL was not associated with any changes in P4 and LH concentrations or in color- Doppler signals of luteal blood flow during the hours of the PGFM pulse. During a PGFM pulse with a peak > 150 pg/mL, P4 decreased between –1 h and 0 h (0 h = peak of pulse), LH increased at 1 h, and luteal blood flow apparently decreased at 2 h of the PGFM pulse. A PGFM pulse with a peak > 150 pg/mL was also associated with a greater peak of an LH pulse after the peak than before the peak of the induced PGFM pulse. These results indicated for the first time that the previously reported temporal associations among the P4 decrease, LH increase, and blood flow changes within the hours of a PGFM pulse is dependent on the prominence of the PGFM pulse.

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CAPÍTULO 4- STUDY 3

ESTUDO ENVIADO PARA PUBLICAÇÃO

Induction of PGFM pulses and luteolysis by sequential estradiol-17β treatments in heifers

G. Pugliesi, M.A. Beg, G.R. Carvalho, O.J. Ginther

Theriogenology, 2011

Induction of PGFM pulses and luteolysis by sequential estradiol-17β treatments in heifers

(Indução de pulsos de PGFM e da luteólise pelo tratamento sequencial com estradiol-17β em novilhas)

G. Pugliesi[a,b,c], M.A. Beg[b], G.R. Carvalho[c], O.J. Ginther[a,b]

[a]Eutheria Foundation, Cross Plains, Wisconsin 53528, Estados Unidos da América

[b]Department of Pathobiological Sciences, University of Wisconsin, Madison, Wisconsin 53706, Estados Unidos da América

[c]Departamento de Zootecnia, Universidade Federal de Viçosa,Viçosa, Minas Gerais, Brasil

RESUMO

Foram estudados os efeitos da indução sequencial de pulsos de PGFM pelo tratamento com estradiol-17β sobre a proeminência dos pulsos de PGFM e na concentração de progesterona (P4) em novilhas. Três tratamentos com veículo (n = 12) ou E2 (n = 12) nas doses de 0,05 mg ou 0,1 mg foram administrados em intervalos de 12 horas, iniciando-se no 15º dia após a ovulação. Amostras de sangue foram colhidas a cada 12 horas entre o 13º e o 24º dia pós-ovulação, e de hora em hora por um período de 12 horas após o primeiro e o terceiro tratamentos. No dia 15 pós-ovulação, todas as novilhas estavam em fase de pré-luteólise e no dia 16 pós-ovulação estavam em pré-luteólise no grupo administrado veículo (n =11) e em pré-luteólise (n = 4) ou luteólise (n = 8) no grupo tratado com E2. A concentração de PGFM no pico dos pulsos de PGFM induzidos durante o período de pré-luteólise no 15º dia pós-ovulação foi maior (P < 0.04) do que os pulsos de PGFM pré-luteolíticos no 16º dia pós-ovulação. O intervalo da ovulação até o início da luteólise foi reduzido (P < 0,05) nas novilhas tratadas com E2 quando comparado as demais novilhas não tratadas (veículo). O pulso de PGFM induzido pelo E2 foi

menos proeminente (P < 0,008) nas novilhas que apresentaram resurgência transitória de P4 do que nas novilhas que apresentaram decréscimo progressivo das concentrações de P4. A hipótese que a exposição repetida ao E2 estimula o aumento da proeminência dos pulsos de PGFM não foi suportada. Ao contrário, a exposição repetida reduziu a proeminência dos pulsos de PGFM em contraste ao estímullo promovido após o primeiro tratamento. Estes resultados indicaram pela primeira vez que a redução na proeminência dos pulsos de PGF2α durante a luteólise pode resultar em resurgimento transitório na concentração de P4.
Palavras-chaves: Estradiol; LH; Luteólise; Novilhas; PGF2α; Progesterona

ABSTRACT

The effects of sequential induction of PGFM pulses by estradiol-17β (E2) on prominence of PGFM pulses and progesterone (P4) concentration were studied in heifers. Three treatments of vehicle (n = 12) or E2 (n = 12) at doses of 0.05 mg or 0.1 mg were given at 12-h intervals beginning on day 15 postovulation. Blood samples were collected every 12 h from days 13 to 24 and hourly for 12 h after the first and third treatments. On day 15, all heifers were in preluteolysis and on day 16 were in preluteolysis in the vehicle-treated heifers (n = 11) and either preluteolysis (n = 4) or luteolysis (n = 8) in the E2-treated heifers. Peak concentration of induced PGFM pulses during preluteolysis on day 15 was greater (P < 0.04) than for pulses during preluteolysis on day 16. The interval from ovulation to the beginning of luteolysis was shorter (P < 0.04) in the E2-treated heifers than in the vehicle-treated heifers. An E2-induced PGFM pulse was less prominent (P < 0.008) in heifers in temporal association with a transient resurgence in P4 than in heifers with a progressive P4 decrease. The hypothesis that repeated E2 exposure stimulates increasing prominence of PGFM pulses was not supported. Instead, repeated exposure reduced the prominence of PGFM pulses in contrast to the stimulation from the first E2 treatment. Results indicated for the first time that reduced prominence of a PGF2α pulse during luteolysis can lead to a transient resurgence in P4 concentration.
Keywords: Estradiol; Heifers; LH; Luteolysis; PGF2α; Progesterone

1. Introduction

Luteolysis consists of functional and structural regression of the CL [1,2]. Functional luteolysis is characterized by a decrease in secretion of progesterone (P4) in sheep [1] and cattle [3]. In heifers, the length of the luteolytic period is 24 h, based on hourly sampling [3]. During spontaneous and induced luteolysis, luteal size and blood flow decrease during functional luteolysis in cattle [4,5] and horses [6]. Structural luteolysis is marked by apoptosis of the luteal cells in ruminants [2,7,8]. Luteolysis in cattle is induced by secretion of PGF2α from the endometrium [1] beginning on day 15, 16, or 17 postovulation [3,4], based on assay of the PGF2α metabolite PGFM. Each pulse of PGFM is several hours in duration [9,10], and sequential pulses of PGF2α are involved in complete luteolysis in cattle [11], horses [12], and sheep [13].

Exposure of the endometrium to the coordinated interactions among P4, estradiol (E2), and oxytocin [14–17] and the uterine expression of their receptors [18,19] are essential for the pulsatile secretion of PGF2α. Exposure of the uterus to P4 for a minimum of 10 days after ovulation is needed to activate the mechanism leading to spontaneous secretion of PGF2α [20–22]. The E2 from developing ovarian follicles is involved in the initiation of luteolysis in cattle [5,23,24] and sheep [25]. In this regard, destruction of ovarian follicles by electrocautery in sheep [26] and x-ray irradiation [23,27] or aspiration of follicle contents in heifers [5] delays, but does not block luteolysis. The relationship between a decrease in E2 by destruction of ovarian follicles and the prominence of the PGFM pulses has not been reported.

In cattle, the prominence of PGFM pulses increases after the transition between preluteolysis and luteolysis [3,10]. Luteolysis is temporally associated with an increase in concentration of E2 [28,29]. Secretion of PGF2α, as represented by PGFM concentration, is induced within 6 to 8 h after a pharmacologic dose (1 or 3 mg) of E2 [5,30]. The prominence of E2-induced PGFM pulses increases with increasing E2 doses as reported in the **Study 2** of the present thesis. A single treatment of 0.1mg of E2 [31,32] given on day 14 postovulation stimulates a prominent pulse of PGFM and reduces the interval from pretreatment ovulation to the end of luteolysis [32]. The effects of

sequential E2 treatment on induction and prominence of sequential PGFM pulses and subsequently on initiation and completion of luteolysis are not known.

Late in preluteolysis and early in luteolysis, P4 decreases during the ascending portion of a PGFM pulse, reaches greatest depression at the peak of the PGFM pulse, and then rebounds during the descending portion [3,10]. A depression in P4 followed by a rebound also occurs within the hours of an E2-induced PGFM pulse [31,33] and during infusion of PGF2α to simulate a spontaneous PGFM pulse [34–35]. The luteolytic effect of PGF2α is balanced against a luteotropic effect of LH pulses during late preluteolysis and early luteolysis [28,37]. The LH concentration increases concomitantly with the P4 rebound that occurs after the peak of a spontaneous [28,37], E2-induced [33], and simulated PGFM pulse [35,36]. The intrapulse changes in P4 and LH concentrations during a PGFM pulse is dependent on prominence of the PGFM pulse as reported in the second study of the present thesis.

The current experiment in cattle aimed to evaluate the following hypotheses: 1) E2 production by the follicles stimulates PGF2α secretion, as indicated by less prominent PGFM pulses and delayed luteolysis after aspiration of follicle contents; and 2) the increasing prominence of PGFM pulses is a function of the continued exposure to E2, as indicated by sequential E2 treatment. In addition, the effects of sequential E2-induced PGF2α pulses on functional and structural luteolysis in cattle was evaluated, and the intrapulse changes in P4 and LH within the hours of an E2-induced PGFM pulse were studied for the first time during both preluteolysis and luteolysis.

2. Materials and methods

2.1. Animals

Twenty-four dairy heifers (Holsteins) aged 18 to 24 mo and weighing 520 ± 17 kg (range: 425 to 615 kg) were used during September and October in the northern temperate zone. Heifers were selected with docile temperament and no apparent abnormalities of the reproductive tract as determined by ultrasound examinations [38]. Animals were handled in accordance with the United States

Department of Agriculture Guide for Care and Use of Agricultural Animals in Research. The day of ovulation was determined by daily transrectal ultrasound examinations [38]. If the heifer presented more than one CL or the single CL was considered undersized (< 3 cm^2 [39]) on day 13 postovulation, the heifer was not used. The heifers were kept under natural light in an open shelter and outdoor paddock and were maintained by *ad libitum* access to a mixture of alfalfa and grass hay, water, and trace-mineralized salt. Heifers remained healthy and in good body condition throughout the experiment.

2.2. Experimental design and treatments

Heifers were assigned randomly by replicate (n = 6) to a vehicle-treated group, a follicle-ablation and vehicle-treated group (FAV), and two follicle-ablation E2-treated groups (FAE-0.05 and FAE-0.1). Heifers in the FAV and FAE treated groups had all follicles ≥ 4 mm ablated once a day from day 13 to day 16 postovulation (Fig. 1). Heifers in the vehicle group and in the FAV group were given an intramuscular treatment of vehicle (2 mL of sesame oil). Heifers in the FAE-0.05 and FAE-0.1 groups were given an intramuscular treatment of 0.05 mg and 0.1 mg, respectively, of E2 in 2 mL of sesame oil. Heifers in all four groups were treated with the vehicle or E2 at 7:00 (Hour 0) and 19:00 (Hour 12) on day 15 postovulation and at 7:00 (Hour 24) on day 16. The number of treatments (three) and the 12-h interval between consecutive treatments were based on the induction of complete luteolysis (P4 < 1ng/mL) after three simulated PGFM pulses by intrauterine infusion of PGF2α at 12-h intervals [11]. The E2 doses were selected according to the reported effectiveness in inducing a PGFM pulse with a single treatment in the **Study 2** of the present thesis. The E2 doses were prepared in the sesame oil vehicle as described [31].

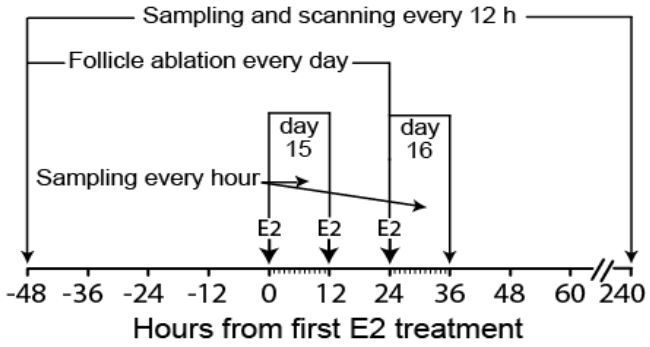

Fig. 1. Diagram of experimental design. Vehicle or doses of estradiol-17β of 0.05 mg or 0.1 mg were given to heifers (n = 6) at 7:00 (Hour 0) and 19:00 (Hour 12) on day 15 postovulation and at 7:00 (Hour 24) on day 16. Follicle ablation was done daily from day 13 to day 16. Blood sampling and scanning at 12-h intervals were done from 7:00 on day 14 (Hour –48) to 7:00 on day 24 (Hour 240). The hourly sampling was done during 12 h for Hours 0 to 12 on day 15 and from Hours 24 to 36 on day 16.

After the first (Hour 0) and the third treatment (Hour 24), a blood sample (10 mL) was taken each hour during a 12-h session from Hours 0 to 12 on day 15 and from Hours 24 to 36 on day 16 (Fig. 1). The hourly sampling was used to study the changes in concentrations of PGFM, P4, and LH and the number, location, and characteristics of pulses of PGFM for each day. An interval of 1 h between blood samples has been recommended for detection of PGFM pulses in cattle [9,32]. The hourly blood samples were collected through an indwelling catheter that was inserted into a jugular vein and secured as described [40]. The catheter was inserted 15 h before collection of the first hourly sample. Heifers had free access to water and hay between sample collections. In addition, blood samples were taken by venipuncture of the coccygeal vein every 12 h from day 13 postovulation until the detection of structural regression of the CL (luteal area, ≤ 2 cm^2) or until day 24 if regression of CL was not detected. The 12-h samples were used to determine the beginning and the end of functional luteolysis in each heifer. The beginning of luteolysis was defined [5] as occurring at the 12-h sample before P4 concentrations decreased ≥ 50 % of the average for the P4 concentrations on days 13 and 14. The end of luteolysis was defined as the 12-h sample when P4 decreased to < 1 ng/mL [4]. The length of luteolysis was the interval between the beginning and end of the luteolysis.

2.3. Follicle ablations

Ultrasound-guided transvaginal ablation of ovarian follicles was performed to remove the follicle source of the circulating E2. Follicle ablation was performed as described [41]. Briefly, a duplex B-mode (gray-scale) and pulsed-wave color Doppler ultrasound instrument (Aloka SSD 3500; Aloka America, Wallingford, CT, USA) equipped with a 5.0-MHz sector and 7.5-MHz linear transducers were used. The diameter of follicles was obtained by transrectal ultrasonography with the linear transducer. Follicle ablation was done during transrectal ovarian manipulation and transvaginal scanning of the follicles with the sector transducer. A 17-gauge needle was connected to a vacuum pump and inserted through the transvaginal transducer and into each follicle ≥ 4 mm. The fluid and inner cells contents of the follicles were removed under vacuum suction (≈130 mm Hg). Caudal epidural anesthesia (2 mL of 2 % lidocaine hydrochloride) was used to minimize abdominal straining and to facilitate the ovarian manipulation. After each procedure, the ovaries were reexamined by transrectal ultrasonography to verify that the contents of all follicles ≥ 4 mm were aspirated.

2.4 Ultrasound scanning

Beginning on day 13 postovulation until the detection of structural regression of CL (luteal area, ≤ 2 cm^2), heifers were scanned every 12 h just after the collection of the coccygeal blood sample. The maximum CL area (cm^2) was determined at each 12-h interval using a B-mode still image and the tracing function. For the CL that showed an anechoic fluid-filled cavity, the area of the cavity was subtracted from the total area [39,42], owing the CL cavity is absent of luteinized cells and does not affect the luteal function [42,43]. Percentage of luteal area with color-Doppler signals of blood flow at each examination was determined as described [4]. All scans were performed at a constant color-gain setting and a velocity setting of 6 cm/sec. The color Doppler-transducer was passed over the entire CL and the images were stored on film. After recording the images from all heifers, the film clips were evaluated to estimate the

percentage of luteal area with color-Doppler signals as described [44]. The operator was unaware of group or hour.

2.5 Identification of PGFM pulses

In each individual heifer, a PGFM increase and decrease (fluctuation) encompassing at least four values including nadirs with a progressive increase on the ascending portion and progressive decrease on the descending portion were evaluated. The CV of PGFM concentrations at the hours of the fluctuation and the intra-assay CV were used to distinguish fluctuations from PGFM pulses as described [4]. When the CV of the values composing a fluctuation of PGFM was at least three times greater than the mean intra-assay CV, the fluctuation was defined as a PGFM pulse. These definitions were based on reports that episodes of PGFM [9,32] at this stage of the estrous cycle can be detected by sampling blood at 1-h intervals. Pulses of PGFM with a peak near the beginning or end of the hourly-sampling session were included in the CV analyses only if they were preceded or followed, respectively, by at least one lower value, but the lower value was not used to calculate nadir concentrations. Nadirs 1 and 2 were defined as the nadirs at the beginning and end of a PGFM pulse, respectively.

The CV-identified PGFM pulses were subgrouped into those that occurred during the preluteolytic period or luteolytic period, based on P4 concentrations in samples taken every 12 h. For the heifers in the FAE-0.05 and FAE-0.1 groups, the PGFM, P4, and LH concentrations were centralized to the peak of the E2-induced PGFM pulses to study the intrapulse changes in P4 and LH during preluteolysis and luteolysis. The PGFM pulses that occurred during preluteolysis for the four experimental groups were subdivided into three different categories to study the effect of the prominence of preluteolytic PGFM pulses on the intrapulse changes in P4 and LH. The prominence categories were based on the concentration at the peak of PGFM pulses of < 50, 50–150, and > 150 pg/mL as described in the **Study 2**.

2.6. End points

The E2 concentrations at the PGFM pulse with the greatest peak on each day (day 15 and day 16) were compared among the four experimental groups (vehicle, FAV, FAE-0.05, and FAE-0.1). Concentrations of PGFM were compared among groups on each day using the mean for all hours of the 12-h session and for the characteristics of the PGFM pulse with the greatest peak during a 12-h session. The PGFM pulse concentrations within a pulse were evaluated at Nadir 1, peak, Nadir 2, and amplitude (difference between peak and Nadir 1). Area under the pulse curve and intervals from treatment (Hour 0 and Hour 24) to Nadir 1 and to the peak and from Nadir 1 to 2 were also considered. The interval from pretreatment ovulation to beginning and end of luteolysis and the length of luteolysis were compared among groups. The P4 concentrations at 12-h intervals were compared among groups for Hours 0 to 60. The P4 concentration, maximum CL area, and CL blood flow centralized to the beginning of luteolysis in each heifer were also compared among groups.

For the heifers with an E2-induced PGFM pulse (FAE-0.05 and FAE-0.1 groups), the PGFM, P4, and LH concentrations for –2 h to 2 h of the PGFM pulse (0 h = pulse peak) were compared between the preluteolytic and luteolytic subgroups regardless of whether of the pulse was on day 15 or 16. The concentration of PGFM, P4, and LH within the hours (–2 h to 2 h) of preluteolytic pulses were compared among three prominence categories (peaks of < 50, 50–150, and > 150 pg/mL).

2.7. Hormone assays

The blood samples were immediately placed in ice water for 10 min after each collection. The samples were centrifuged (2000 X g for 10 min) and the plasma was decanted and stored (–20 ºC) until assayed. The plasma samples were assayed for PGFM by an ELISA that was developed and validated in our laboratory for use with bovine plasma [10]. Plasma P4 concentrations were assayed with a solid-phase RIA kit containing antibody-coated tubes and ^{125}I-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA). The plasma concentrations of LH were determined by RIA

as described [45] and modified [46] for bovine plasma in our laboratory. Plasma E2 concentrations were assayed using a commercial RIA kit (Double Antibody Estradiol: Diagnostic Products Corporation) as reported [47]. The intra- and interassay CV and sensitivity for PGFM, respectively, were 7.3 %, 8.2 %, and 14.0 pg/mL. The intra-assay CV and sensitivity for P4, LH, and E2, respectively, were 6.2 % and 0.07 ng/mL, 8.4 % and 0.03 ng/mL, and 4.0 % and 0.06 pg/mL.

2.8. Statistical analysis

Outlying observations greater than three interquartile ranges from the mean were not used in the statistical analyses (PROC UNIVARIATE; SAS online Doc 9.1.3; SAS, Cary, NC, USA); two LH outliers were found and removed. Data that were not normally distributed by the Shapiro-Wilk test were transformed to natural logarithms, square roots, or ranks. When there was an apparent disparity among groups at Hour 0 or at the beginning of luteolysis, the percentage change from the first hour or a covariance analysis with the concentrations at Hour 0 as the covariate was used. The differences for PGFM concentrations were analyzed for the main effect of group and hour and for the interaction of group by hour on each day (days 15 and 16). The SAS PROC MIXED procedure (Version 9.2; SAS) was used with a REPEATED statement to account for autocorrelation between sequential measurements. The Least Significant Difference (LSD) test was used when comparisons between hours were made within a group or among more than two means. The P4 concentrations, maximum CL area, and CL blood flow were compared among groups in a group-by-hour analysis. The LSD test was used to locate differences among groups and among hours within each group. Hormone concentrations during E2-induced PGFM pulses were compared between preluteolytic and luteolytic periods in a period-by-hour analysis using the peak (0 h) of a PGFM pulse as a centralized reference point for P4 and LH. The LSD test was used to locate differences among hours within each period. The comparisons among the prominence categories of PGFM pulses for PGFM, P4, and LH concentrations during a PGFM pulse were made in a category-by-hour analysis. The LSD test was used to locate differences among categories and among hours within each category. Discrete data for characteristics and

intervals for PGFM pulses were analyzed for differences among groups in each day by the LSD test and for differences between days in each group by Student's unpaired *t*-tests. A Fischer's exact test was used for comparison of the frequency of pulses among groups. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $P > 0.05$ to $P \leq 0.1$ indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise indicated.

3. Results

The probabilities for main effects and an interaction for the factorial analyses are given in the figures and the probabilities for differences in discrete end points are given in the tables or text. There was a difference ($P < 0.0001$) among the four groups in the mean E2 concentration at the hour of the peak of PGFM pulses on day 15 postovulation as follows: vehicle (0.52 ± 0.16 pg/mL^{c}), FAV (0.36 ± 0.03 pg/mL^{c}), FAE-0.05 (3.09 ± 0.41 pg/mL^{b}), and FAE-0.1 (6.91 ± 1.87 pg/mL^{a}); means with a different superscript are different ($P < 0.05$). For day 16, differences ($P < 0.0002$) among groups for the E2 concentration at the hour of the peak of a PGFM pulse were as follows: vehicle (0.65 ± 0.20 pg/mL^{b}), FAV (0.42 ± 0.05 pg/mL^{b}), FAE-0.05 (3.07 ± 0.62 pg/mL^{a}), and FAE-0.1 (5.41 ± 1.86 pg/mL^{a}).

The number of heifers with CV-identified pulses of PGFM with a peak > 150 pg/mL was greater ($P < 0.01$) in the FAE-0.05 and FAE-0.1 groups (6 of 6 heifers in each group) than in the vehicle (2 of 6) and FAV (0 of 6) groups on day 15, but there were no differences among groups on day 16 (3 of 6 heifers in the FAE-0.05 group; and 1 of 6 heifers in each of the other three groups). The number of heifers with a PGFM peak > 150 pg/mL was greater ($P < 0.05$) on day 15 than on day 16 in each E2-treated group.

On day 15, PGFM concentrations at the peak, amplitude, and area under curve of the PGFM pulse were greater ($P < 0.004$), and the interval from Nadir 1 to Nadir 2 was longer ($P < 0.01$) in the FAE-0.05 and FAE-0.1 groups than in the vehicle and FAV groups (Table 1). On Day 16, the only significant difference among the groups was a shorter ($P < 0.05$) interval from Nadir 1 to Nadir 2 in

the FAE-0.1 group than in the FAE-0.05 group. The interval from treatment to Nadir 1 and to the peak of the PGFM pulse did not differ among groups on either days 15 or 16 (data not shown). The PGFM pulse concentrations in the FAE-0.05 and FAE-0.1 groups were greater ($P < 0.05$) on day 15 than on day 16 at the peak, amplitude, and area under curve. The interval from Nadir 1 to Nadir 2 in the FAE-0.1 group was longer ($P < 0.05$) on day 15 than on day 16. There were no other significant differences between days for either group.

Table 1. Mean ± SEM characteristics of PGFM concentrations in a vehicle group, and in three groups with ovarian follicles ablated (FA) from days 13 to 16 postovulation and treated three times every 12 h starting on day 15 with doses of 0.0 (FAV), 0.05 or 0.1 mg estradiol-17β (E).

End point	day	Group			
		Vehicle	FAV	FAE-0.05	FAE-0.1
No. of heifers		6	6	6	6
No. of heifers with a PGFM pulse	15	5	5	6	6
	16	5	4	5	4
Mean PGFM/12-h * (pg/mL)	15	26.7 ± 6.7^{b}	28.0 ± 2.0^{b}	78.3 ± 8.9^{a,x}	89.6 ± 11.0^{a,x}
	16	34.9 ± 11.1	34.1 ± 3.6	42.7 ± 7.3^{y}	27.5 ± 8.0^{y}
PGFM pulse concentrations (pg/mL):					
Peak	15	106.8 ± 44.1^{b}	63.1 ± 2.9^{b}	274.0 ± 37.4^{a,x}	273.5± 44.6^{a,x}
	16	128.7 ± 60.3	89.4 ± 35.0	174.2 ± 35.6^{y}	95.1 ± 44.4^{y}
Nadir 1†	15	21.1 ± 3.4	15.1 ± 5.0	26.2 ± 4.9^{x}	19.2 ± 4.5
	16	15.9 ± 8.2	24.6 ± 8.5	14.5 ± 3.2^{y}	26.6 ± 9.3
Nadir 2 †	15	14.7 ± 3.4	16.6 ± 3.4	20.1 ± 5.7	35.1 ± 14.1
	16	17.4 ± 7.6	19.6 ± 6.0	24.6 ± 8.5	20.3 ± 7.5
Amplitude‡	15	74.1 ± 58.5^{b}	48.9± 2.7^{b}	247.8 ± 34.0^{a,x}	254.2 ± 43.6^{a,x}
	16	113.0 ± 81.7	64.9 ± 38.7	159.7± 33.6^{y}	68.5 ± 38.0^{y}
Area under pulse curve	15	165.3 ± 87.2^{b}	85.2 ± 15.3^{b}	655.7 ± 117.3^{a,x}	692.0 ± 133.5^{a,x}
	16	226.8 ± 118.1	129.2 ± 68.1	281.4 ± 50.0^{y}	105.3 ± 38.8^{y}
Interval from Nadir 1 to Nadir 2 (h)	15	4.3 ± 0.5^{b}	4.0 ± 0.7^{b}	6.5 ± 0.6^{a}	7.0 ± 0.8^{a,x}
	16	4.0 ± 0.7^{ab}	4.5 ± 0.6^{ab}	5.6 ± 1.0^{a}	3.3 ± 0.3^{b,y}

* Blood sampling was done hourly for Hours 0 to 12 on day 15 postovulation and Hours 24 to 36 on day 16 (Hour 0= first treatment)

† Nadirs 1 and 2 are the nadirs at the beginning and the end of a pulse, respectively.

‡ Amplitude is the difference in concentration between Nadir 1 and peak.

^{ab} Means within a row without a common superscript are different between groups ($P < 0.05$).

^{xy} Means within a column and end point with different superscripts are different between days ($P < 0.05$).

There were no differences or approaching differences in the discrete characteristics of PGFM pulses between vehicle and FAV groups or between the two E2-treated groups. The groups were therefore combined into control heifers (vehicle and FAV) and E2-treated heifers (FAE-0.05 and FAE-0.1) but only for study of the effect of E2 on PGFM. Each heifer on day 15 was in preluteolysis. The concentrations of PGFM for the 12-h session of hourly sampling on day 15 had significant combined-group and hour effects and an interaction of combined-group by hour (Fig. 2). The combined-group effect represented greater PGFM concentrations averaged over the 12 h in the E2-treated heifers (83.9 ± 7.4 pg/mL) than in the control heifers (27.4 ± 2.5 pg/mL). The interaction was primarily from an increase in PGFM concentration between Hours 2 and 7 in the E2-treated heifers, compared to no difference among hours in the control heifers.

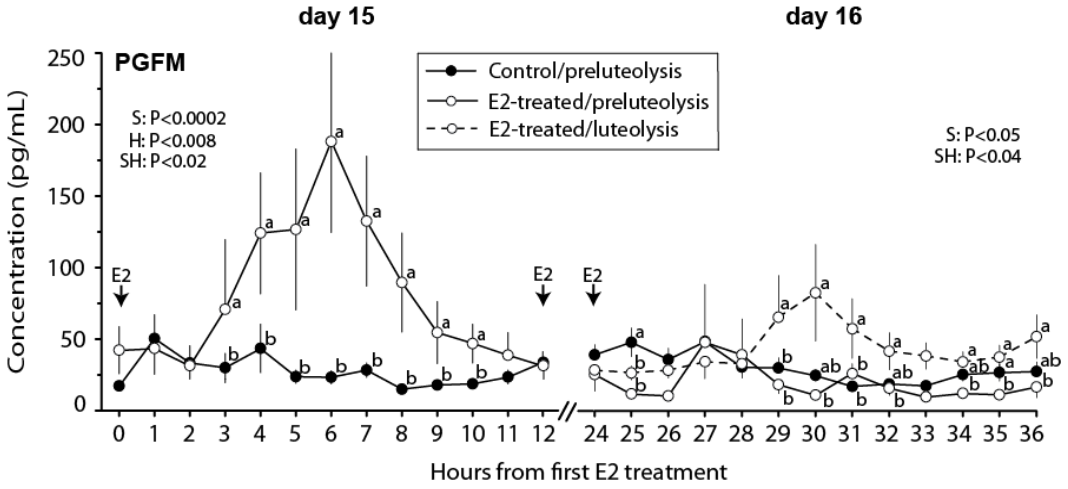

Fig. 2. Mean ± SEM for concentrations of PGFM from first treatment (Hour 0) on day 15 in control and E2-treated heifers, and on day 16 in a control/preluteolysis, E2-treated/preluteolysis and E2-treated/luteolysis subgroups. Main effects of subgroup (S), hour (H) and their interaction (SH) that were significant are shown. Hour of a difference ($P < 0.05$) in PGFM among subgroups is indicated by different letters.

On day 16, only one of 12 control heifers (vehicle and FA groups) was in luteolysis and was not included in the analyses. Eight of 12 E2-treated heifers were in luteolysis at the hour of the third treatment (Hour 24), and heifers were partitioned into three subgroups: control/preluteolysis (n = 11), E2-treated/preluteolysis (n = 4), and E2-treated/luteolysis (n = 8). A subgroup effect and an interaction of subgroup by hour were found (Fig. 2). The subgroup effect reflected the following PGFM concentrations averaged over the hourly samples

for the 12-h sessions: control/preluteolysis subgroup (34.5 ± 3.4 pg/mL^{ab}), E2-treated/preluteolysis subgroup (19.5 ± 3.8 pg/mL^{b}), and E2-treated/luteolysis subgroup (42.9 ± 4.5 pg/mL^{a}). The interaction reflected an increase in PGFM concentration between Hours 28 and 30 in the E2-treated/luteolysis subgroup.

For day 15, PGFM concentration during the PGFM pulses from –2 h to 2 h (0 h = peak) for the control and E2-treated heifers showed heifer and hour effects (Fig. 3). The PGFM concentration averaged over –2 h to 2 h of the PGFM pulse was greater in the E2-treated heifers (130.1 ± 11.6 pg/mL) than in the control heifers (39.1 ± 5.5 pg/mL) and greater at the hour of the peaks of PGFM pulses, as expected from centralizing to the peak. For day 16, significant subgroup and hour effects and an interaction of subgroup by hour were detected. The subgroup effect reflected the following PGFM concentration averaged over –2 h to 2 h: control/preluteolysis subgroup (39.9 ± 5.0 pg/mL^{ab}), E2-treated/preluteolysis subgroup (34.5 ± 12.6 pg/mL^{b}), and E2-treated/luteolysis subgroup (73.1 1 ± 12.6 pg/mL^{ab}). The subgroup-by-hour interaction represented greater ($P < 0.007$) PGFM concentrations at –1 h and 0 h of a PGFM pulse in the E2-treated/preluteolysis subgroup than in the other two subgroups.

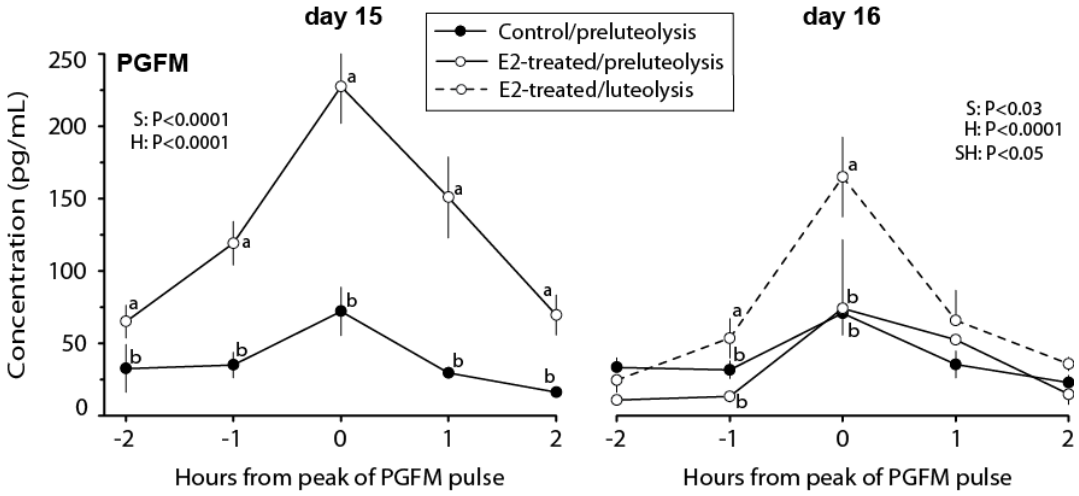

Fig. 3. Mean ± SEM intrapulse concentration of PGFM centralized to the peak of the PGFM pulse (0 h) on day 15 in control and E2-treated heifers and on day 16 in a control/preluteolysis, E2-treated/preluteolysis and E2-treated/luteolysis subgroups. Main effects of subgroup (S), hour (H), and their interaction (SH) that were significant are shown. Hour of a difference ($P < 0.05$) in PGFM among subgroups is indicated by different letters.

For the analyses that involved blood sampling at 12-h intervals, one heifer in each of the FAV and FAE-0.1 groups was omitted owing to a presence of a luteinized follicle. The beginning of functional luteolysis was not detected in any heifer by day 15. The intervals from pretreatment ovulation to beginning and end of functional and structural luteolysis were shorter ($P < 0.04$) in the FAE-0.05 and FAE-0.1 groups than in the FAV group (Table 2). The interval from beginning to end of functional luteolysis was longer ($P < 0.02$) in the FAE-0.05 group than in the other three groups. The P4 concentrations in samples collected at 12-h intervals and examined by covariance analysis had a significant group effect (Fig. 4) that reflected the following P4 concentrations averaged over Hours 0 to 60: vehicle (8.3 ± 0.8 ng/mL^{ab}), FAV (11.2 ± 0.9 ng/mL^{a}), FAE-0.05 (4.9 ± 0.4 pg/mL^{b}), and FAE-0.1 (5.1 ± 1.0 ng/mL^{b}). The hour effect was from a gradual decrease in P4 between Hours 0 and 60, averaged over all groups. Although there was no interaction, the P4 concentration began to decrease at Hour 12 in the FAE-0.05 and FAE-0.1 groups and at Hour 48 in the vehicle group; an hour effect was not detected in the FAV group.

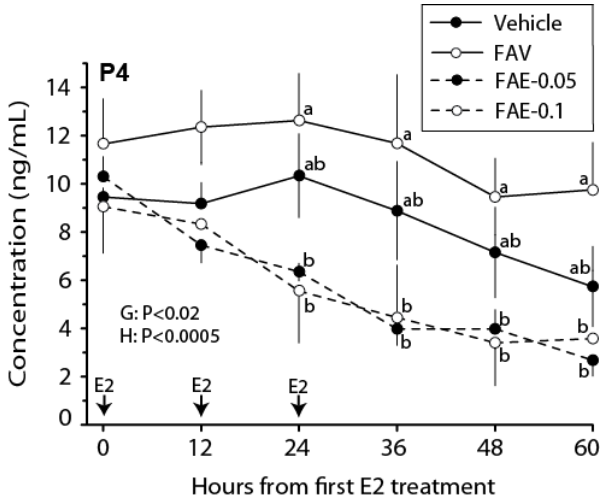

Fig. 4. Mean ± SEM progesterone (P4) concentrations at 12-h intervals in a vehicle group, follicle-ablated and vehicle-treated group (FAV), and in two follicle-ablated groups treated with three doses of estradiol-17β of 0.05 mg (FAE-0.05) or 0.1 mg (FAE-0.1) on days 15 and 16 postovulation (n = 6 heifers/group). Main effects of group (G) and hour (H) that were significant are shown. Hour of a difference ($P < 0.05$) in P4 among groups is indicated by different letters. The first decrease ($P < 0.05$) between two hours in P4 concentrations in each group is indicated by an asterisk between the two hours.

Table 2. Mean ± SEM length of intervals between events associated with luteolysis in a vehicle group and in three groups with ovarian follicles ablated (FA) from days 13 to 16 postovulation and treated three times every 12 h starting on day 15with doses of 0.0 (FAV), 0.05 or 0.1 mg estradiol-17β (E).

End point	Group			
	Vehicle	FAV	FAE-0.05	FAE-0.1
No. of heifers	6	5	6	5
Intervals:				
Ovulation to beginning of functional luteolysis (d)*	17.8 ± 0.6[ab]	18.5 ± 0.4[a]	16.1 ± 0.1[b]	16.5 ± 0.7[b]
Ovulation to end of functional luteolysis (d)	19.0 ± 0.6[ab]	20.0 ± 0.4[a]	18.3 ± 0.4[b]	17.8 ± 0.6[b]
Beginning to end of functional luteolysis (h)	30.0 ± 2.7[b]	36.0 ± 5.4[b]	54.0 ± 6.8[a]	31.2 ± 4.8[b]
Ovulation to structural luteolysis (d)†	19.5 ± 0.6[ab]	20.4 ± 0.5[a]	18.5 ± 0.3[b]	18.6 ± 0.5[b]

* Beginning of functional luteolysis was defined as the 12-h sample after ovulation before plasma P4 declined to less than 50% of the average for the P4 concentrations on days 13 and 14 postovulation.

† Structural luteolysis was defined as the 12-h evaluation after ovulation when the maximum CL area was ≤ 2 cm^2.

[ab] Means within a row without a common superscript are different between groups ($P < 0.05$).

A progressive decrease in P4 concentrations from the beginning to the end of functional luteolysis was detected in each heifer in the vehicle, FAV, and FAE-0.1 groups. Three heifers in the FAE-0.05 group had a progressive decrease in P4 and three had a transient increase or resurgence and were designated as subclasses and analyzed separately (Fig. 5). A significant hour effect and an interaction of hour by subclass were detected. The interaction reflected a transient increase in P4 during Hours 36 to 48 in the subclass with a transient resurgence but not in the subclass with a progressive P4 decrease. The PGFM pulse with the greatest peak in each heifer did not differ between the two subclasses on day 15 (Hours 0 to 12), but on day 16 (Hours 36 to 48) was greater ($P < 0.008$) in the subclass with a progressive decrease in P4 (224.6 ± 28.9 pg/mL) than in the subclass with a transient resurgence (65.7 ± 36.8 pg/mL).

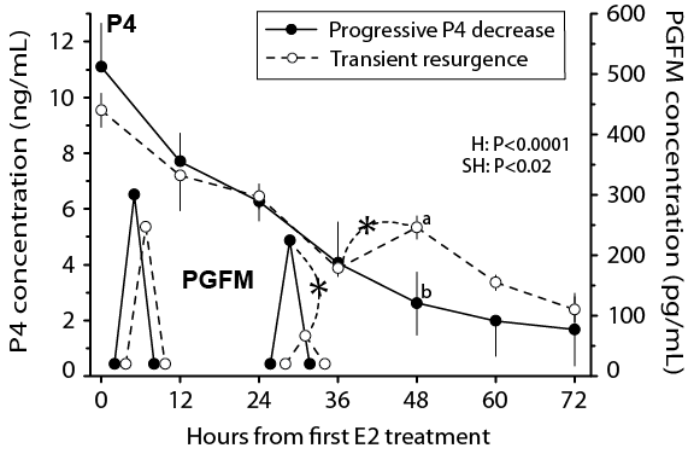

Fig. 5. Mean ± SEM progesterone (P4) concentrations at 12-h intervals, and location and concentration of the peak of a PGFM pulse on days 15 or 16 postovulation in a follicle-ablated group treated with three times with estradiol-17β at a dose of 0.05 mg (FAE-0.05) at 12-h intervals on days 15 and 16. The heifers were sub-classified into those with a progressive decrease in P4 after beginning of luteolysis (n = 3) or in those with a transient P4 resurgence (n = 3). Main effects of hour (H) or interaction of subclass by hour (SH) that were significant are shown. Hour of a difference (P < 0.004) in P4 between the subclasses of heifers is indicated by different letters. An increase (P < 0.05) between two hours in P4 concentrations are indicated by an asterisk between two hours. A difference in PGFM concentrations at the peak of a PGFM pulse between the subclasses of heifers in each day is indicated by an asterisk between the two peaks.

The P4 concentrations, CL area, and CL blood flow at 12-h intervals from the beginning of functional luteolysis are shown (Fig. 6). For the P4 concentrations, there were group and hour effects and an interaction of group by hour. The group effect represented greater P4 concentration in the FAE-0.05 group than in the other three groups. The interaction represented greater P4 concentration from 24 to 48 h after the beginning of luteolysis in the FAE-0.05 group than in the other three groups. For CL area (cm^2), there was only an hour effect, as expected from using the beginning of luteolysis as the reference point. There was disparity among groups in percentage of CL area with color-Doppler signals of blood flow at the beginning of luteolysis. Therefore, data were converted to the percentage change from beginning of luteolysis in each heifer. The percentage change in CL blood flow showed group and hour effects and an interaction of group by hour. The group effect represented the following percentage change in CL area with color signals of blood flow: vehicle (–66.8 ± 13.3 $\%^{b}$), FAV (–32.3 ± 15.6 $\%^{ab}$), FAE-0.05 (–3.4 ± 7.7 $\%^{a}$), and FAE-0.1 (–45.0 ± 10.5 $\%^{b}$). The interaction represented the beginning of a decrease in CL blood flow during the first 12 h of functional luteolysis in the vehicle and

FAE-0.1 groups and at 24 to 36 h in the FAV group; there was no effect of hour in the FAE-0.05 group when each group was analyzed separately.

The PGFM, P4, and LH concentrations within E2-induced PGFM pulses during the preluteolytic and luteolytic periods are shown (Fig. 7). The total number of PGFM pulses in the preluteolytic period was 18 pulses on day 15 and three pulses on day 16. There were eight PGFM pulses in the luteolytic period on day 16. The PGFM concentration showed period and hour effects but no interaction. The period effect represented greater concentrations averaged over –2 h to 2 h in the preluteolytic period (127.9 ± 11.1 pg/mL) than in the luteolytic period (76.9 ± 11.9 pg/mL). For P4 concentration centralized to the PGFM peak (0 h), period and hour effects and an interaction of period by hour were detected. The hour effect represented a decrease in P4 concentration between –2 h to 0 h. The interaction represented a decrease in P4 concentration between –1 h and 1 h followed by an increase between 1 h and 2 h in the preluteolytic period and a decrease between –2 h to 0 h without an increase in the luteolytic period. For the LH concentrations, a period effect was significant and an hour effect approached significance. The period effect represented lower LH concentrations in the preluteolytic period (0.29 ± 0.02 ng/mL) than in the luteolytic period (0.47 ± 0.05 ng/mL). The hour effect was primarily from an increase in LH between –2 h and 2 h of the PGFM pulse. Although the interaction was not significant, a paired t-test indicated that LH concentration increased ($P < 0.05$) between –2 h and 2 h in the preluteolytic period and between –1 h and 1 h in the luteolytic period.

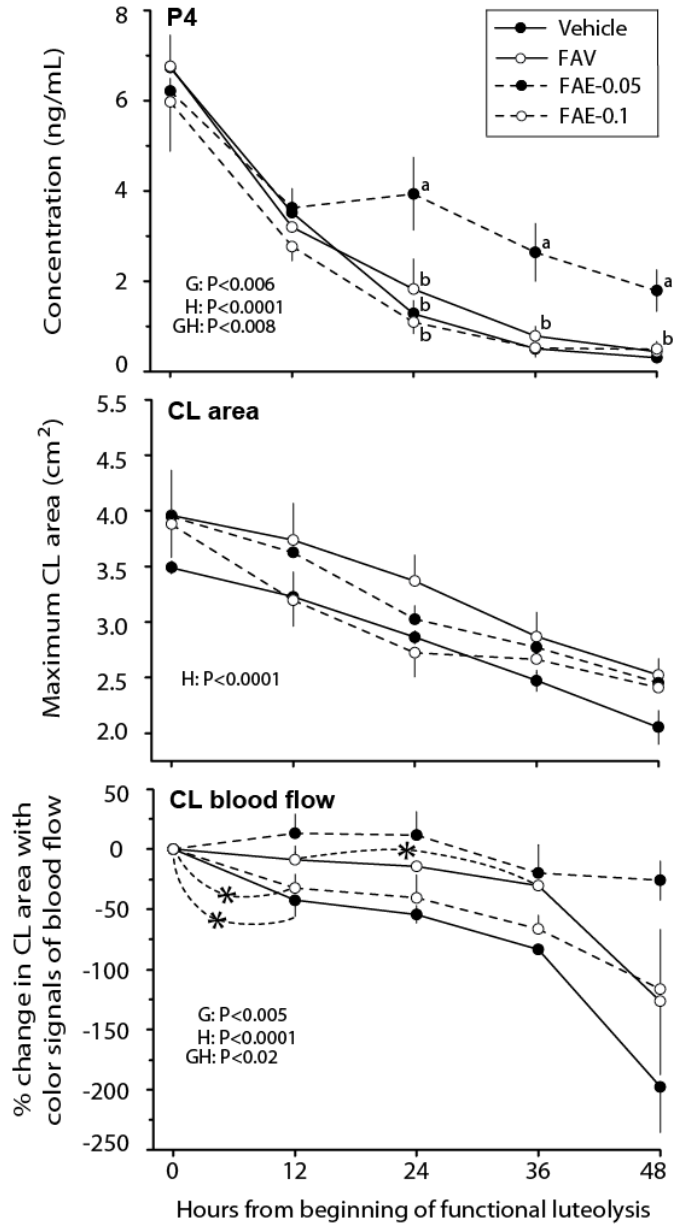

Fig. 6. Mean ± SEM of progesterone (P4) concentrations, maximum CL area, and percentage change in luteal area with signals of blood flow at 12-h intervals centralized to the beginning of functional luteolysis in a vehicle group, in a follicle ablated and vehicle-treated group (FAV), and in two follicle ablated groups treated with three doses of estradiol-17β of 0.05 mg (FAE-0.05) or 0.1 mg (FAE-0.1) at 12-h intervals on days 15 and 16 postovulation (n = 6 heifers/group). Main effects of period (G) and hour (H) and their interaction (GH) that were significant are shown. Hour of a difference ($P < 0.05$) in P4 is indicated by different letters. The first decrease ($P < 0.05$) between two hours in blood flow in each group is indicated by an asterisk between the two hours.

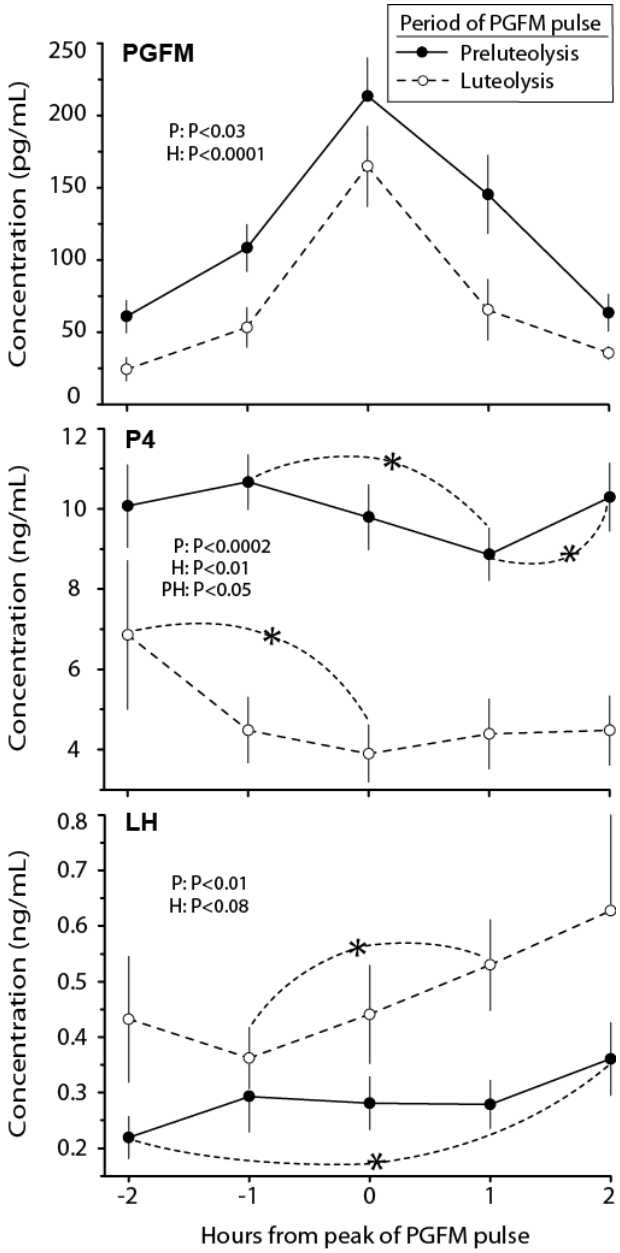

Fig. 7. Mean ± SEM intrapulse concentration of PGFM, P4, and LH centralized to the peak of a PGFM pulse induced by estradiol-17β during the preluteolytic and luteolytic periods. Main effects of period (P) and hour (H) and their interaction (PH) that were significant are shown. A decrease or increase ($P < 0.05$) between two hours in P4 or LH concentrations are indicated by an asterisk between the two hours.

The PGFM, P4, and LH concentrations within the hours of PGFM pulses are shown for the three prominence categories of < 50, 50–150, and > 150 pg/mL at the PGFM peak during preluteolysis (Fig. 8). For P4 concentration centralized to the PGFM peak (0 h), the interaction was not significant, but a difference ($P < 0.05$) among hours was detected only for the >150 category and involved a decrease ($P < 0.05$) between –1 h and 1 h followed by an increase

between 1 h and 2 h of the PGFM pulse. Although a main effect of category or hour or an interaction were not detected for LH concentrations, a difference ($P < 0.03$) among hours was detected only for the >150 category and involved an increase ($P < 0.05$) between –1 h and 2 h of the PGFM pulse.

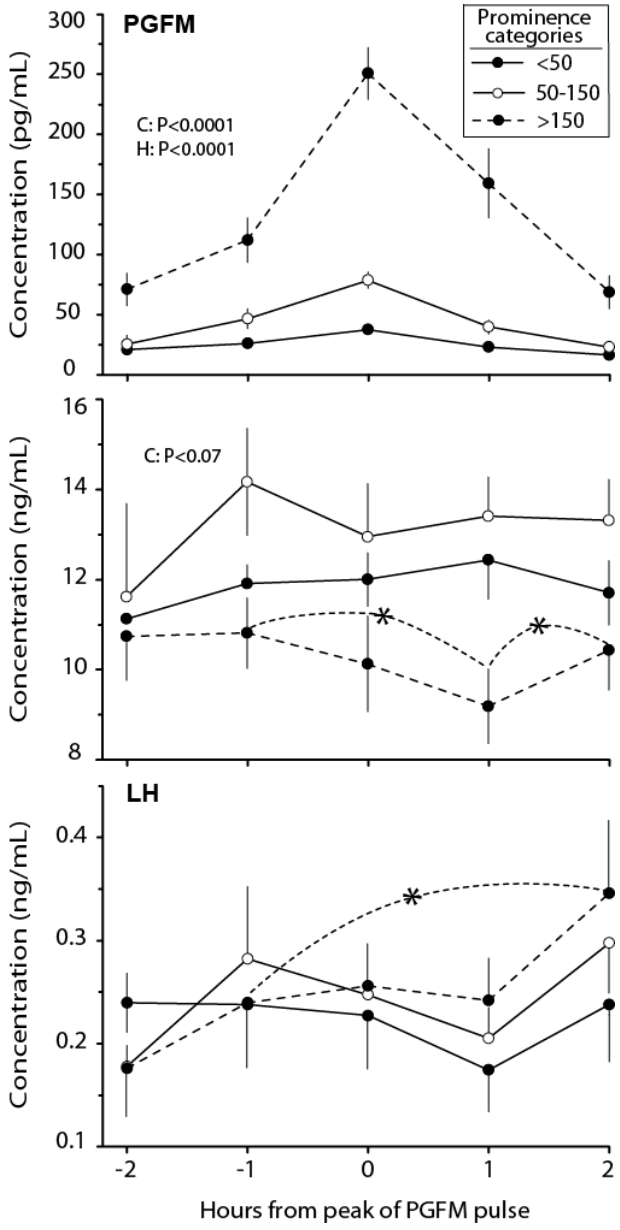

Fig. 8. Mean ± SEM intrapulse concentration of PGFM, P4, and LH during preluteolysis centralized to the peak of PGFM pulses in three categories of prominence (pg/mL at peak) of PGFM pulses (<50, n =12 pulses; 50–150, n = 14 pulses; and >150, n= 18 pulses). Main effects of category (C) and hour (H) that were significant are shown. A decrease or increase ($P < 0.05$) between two hours in P4 or LH concentrations is indicated by an asterisk between the two hours.

4. Discussion

Reduction of E2 concentrations by ablation of follicles on days 15 and 16 postovulation was unsuccessful. That is, there was no difference in E2 concentrations at the peak of a PGFM pulse between heifers with intact and ablated follicles. Apparently, the growing follicles of a follicular wave at this stage were not producing an adequate amount of E2 to result in an E2 reduction after ablation. Previous reports indicate that E2 concentrations in the follicular fluid [48], systemic circulation [49], and vena cava [50] do not begin to increase until the largest follicle of the emerging wave reaches 8.5 mm. Ablation of all follicles ≥ 4 mm when the largest follicle is 8.5 mm reduces the E2 concentrations [50]. Hypothesis 1 that E2 has a positive effect on the prominence of PGFM pulses and the initiation of luteolysis was not testable by consecutive aspiration of follicle contents on days 13 to 16.

The E2 concentration at the peak of a PGFM pulse in the two E2-treated groups seemed similar to reported concentrations during an E2 preovulatory surge [45,49] and was proportionately greater for the 0.1 mg dose than for the 0.05 mg dose as expected. The absence of a difference between doses in the prominence of a PGFM pulse appears to conflict with the results in the **Study 2** on day 14 that the prominence of E2-induced PGFM pulses was greater with the 0.1 mg dose than with the 0.05 mg dose. The reported dose response to E2 on day 14 and the lack of a dose response on day 15 in the current study may be related to an increase between days 14 and 15 in the sensitivity of uterine response to E2 concentrations. An increase in the expression of mRNA for E2 receptors after day 14 has been reported [51]. In this regard, a single treatment with 0.01 mg E2 did not stimulate a PGFM response on day 10 but elicited an intermediate positive response on day 12 and a greater response on day 14 [10]. Prolonged exposure of the endometrium to P4 is needed for the expression of E2 receptors [16,21,22].

Administration of E2 one to several days before the expected beginning of luteolysis has been used previously to induce PGF2α secretion [5,30]. Treatment on day 14 induces a PGFM pulse [31–33]. The current study differs in that the E2 treatment was at Hours 0 and 12 on day 15 and Hour 24 on day 16, whereas treatment was only on day 14 in the previous studies. All heifers in

the current study were in preluteolysis at the first treatment on day 15 (Hour 0). Hourly samples were not taken after the second E2 treatment (Hour 12). The P4 in the samples at 12-h intervals indicated that 75 % of E2-treated heifers were in luteolysis at the hour of the third treatment compared to 8 % in the control heifers. This allowed for novel comparisons of the PGFM response to E2 between heifers in preluteolysis and heifers in luteolysis.

The PGFM response to the third E2 treatment on day 16 (Hour 24) was represented by greater reduction in hourly PGFM concentrations and in prominence of the identified PGFM pulses than for the first E2 treatment on day 15. This result was contrary to Hypothesis 2 in that sequential E2 treatments during preluteolysis resulted in reduced prominence of the PGFM pulses rather than increased prominence. The PGFM increase on day 16, however, was greater in heifers in luteolysis than in the heifers in preluteolysis. The differences between the preluteolytic and luteolytic E2-treated subgroups on day 16 were expressed at Hour 30 (approximate location of the induced PGFM pulse) and at –1 h and 0 h (peak) of the PGFM pulse. These responses seemed complex in that the differences were between days 15 and 16 and between preluteolytic and luteolytic periods on day 16.

Our favored interpretation is based on three considerations: (1) Exposure of heifers to exogenous E2 in the current study resulted in circulating E2 concentrations that were comparable to the reported concentrations from secretion of E2 during a preovulatory E2 surge [45,49]. (2) Spontaneous PGFM pulses in heifers are more prominent during luteolysis than during preluteolysis and postluteolysis [3,10]. (3) Endogenous E2 concentrations increase during the progression from preluteolysis to luteolysis [28,29] to postluteolysis [45,52]. The interpretation is as follows: (1) The induction of a prominent PGFM pulse in the preluteolytic heifers on day 15 represents an acute response. (2) The greatly reduced PGFM response in the preluteolytic heifers on day 16 represents the repeated exposure to E2 from the three treatments at 12-h intervals, similar to the reduced prominence of spontaneous PGFM pulses during postluteolysis [3,10] when E2 also is chronically elevated [52]. (3) The greater PGFM response on day 16 in the luteolytic heifers than in the preluteolytic heifers represents the greater prominence of PGFM pulses during spontaneous luteolysis than during preluteolysis, owing to increased E2

secretion; however, the PGFM response in the heifers in luteolysis was not as great as for a spontaneous pulse during luteolysis because of the overriding high concentrations of exogenous E2. This interpretation supports the concept that E2 is a fundamental factor in the spontaneous burst of PGF2α secretion as represented by a PGFM pulse. The interpretation also considers for the first time that E2 accounts for the varying prominence of PGFM pulses during preluteolysis, luteolysis, and postluteolysis and that E2 may have a biphasic effect on secretion of PGF2α. The biphasic effect is an initial acute stimulatory effect and then an inhibitory effect after repeated or chronic exposure.

The association of a chronic elevation of E2 and the greatly reduced PGFM response on day 16 in the two E2-treated groups may represent a down-regulation of oxytocin receptors in the uterus. In this regard, the high concentrations of E2 during the preovulatory period in cattle results in loss of uterine oxytocin receptors and reduction in receptor activity [53]. In bovine endometrial cell culture, E2 induces a short-term up-regulation of oxytocin receptors after a priming effect of P4, but the effect is not maintained for more than 1 to 2 d [54]. The binding of oxytocin to endometrial oxytocin receptors is involved in the pulsatile secretion of PGF2α [55,56]. A study on the expression of oxytocin receptors and secretion of PGF2α pulses by a chronic E2 increase at different concentrations is indicated.

Functional luteolysis initiated by the sequential E2-induced PGF2α pulses was indicated by the lower P4 concentrations in the samples at 12-h intervals and the shorter interval from pretreatment ovulation to beginning and end of functional luteolysis in the two E2-treated groups than in the FAV group. An effect of E2 on induction of structural luteolysis was indicated by the shorter interval from pretreatment ovulation to the structural luteolysis (CL area, < 2 cm^2) in the two E2-treated groups than in the FAV group. It is unknown if structural luteolysis was directly affected by E2 or was associated with or a consequence of functional luteolysis. Structural luteolysis is characterized by changes not only in the luteal (steroidogenic) cells as occurs during functional luteolysis, but also in the non-luteal (accessory) cells of the CL [7,57]. The effects of PGF2α on the CL cells are mediated by local factors such as endothelin-1, cytokines, vasoactive peptides, and nitric oxide, which lead to the apoptosis process [8,58,59].

A novel observation was that functional luteolysis (P4 decrease) in the group treated with 0.05 mg E2 was unlike luteolysis in the group treated with 0.1 mg or spontaneous luteolysis in the vehicle and FAV groups. This was indicated by the longer length of luteolysis and the greater concentrations in P4 at 24 h, 36 h, and 48 h after the beginning of functional luteolysis in the FAE-0.05 group than in the other three groups. The difference in luteolysis in the FAE-0.05 group was attributable to the three heifers with a transient P4 resurgence at Hour 48. The PGFM pulses were less prominent on day 16 in the heifers with transient resurgence. This result suggests that an undersized sequential PGFM pulse or absence of a pulse at the appropriate time may result in an increase in P4 production by the luteal cells thereby interrupting the luteolytic process (resurgence). The inability to maintain the decrease in P4 production in the FAE-0.05 group is consistent with the absence of an intrapulse decrease in P4 concentrations during the hours of a PGFM pulse of intermediate prominence (peak, 50–150 pg/mL), as found in the current studies (**Study 2** and **3**). The delay in completion of luteolysis in the FAE-0.05 group also is consistent with a longer length of luteolysis in heifers with luteolytic PGFM pulses dimished by a prostaglandin inhibitor as observed in the **Study 1**. In this regard, completion of luteolysis (P4 < 1ng/mL) occurs after three sessions of PGF2α infusion 12 h apart to simulate sequential PGFM pulses but resurgence occurs after only one session [11]. In addition, a decrease followed by a resurgence in P4 in heifers treated with a single intrauterine PGF2α infussion to simulate a PGFM pulse was temporally related to a greater CL area than in heifers with a progressive decrease in P4 from sequential simulated PGFM pulses [11]. In the current study, the transient resurgence in functional luteolysis was temporally related to maintainance of luteal blood flow, as indicated by a decrease in the percentage change in CL area with color-Doppler signals of blood flow within 48 h after the beginning of luteolysis in the vehicle, FAV, and FAE-0.1 groups but not in the FAE-0.05 group. In this regard, luteal blood flow is considered to be a key regulatory component of CL function in cattle [59,60,61].

A decrease in P4 concentration followed by a complete rebound and an increase in LH concentration are temporally related to spontaneous [3,6], E2-induced [31,33], and simulated [35,36] PGFM pulses during preluteolysis. The decrease in P4 and increase in LH also occurs during a spontaneous PGFM

pulse during luteolysis, but the rebound in P4 does not reach the prepulse concentrations [3,28]. In the current study, an E2-induced PGFM pulse was temporally associated with an increase in LH concentration within 2 h after the pulse peak during preluteolysis and luteolysis. However, the increase in LH during the hours of a PGFM pulse was temporally associated with a complete rebound in P4 during the preluteolysis and an incomplete rebound during the luteolysis. These intrapulse changes in P4 during E2-induced preluteolytic and luteolytic PGFM pulses were similar to the P4 changes during spontaneous PGFM pulses [10,28]. This is the first report using E2-induced PGFM pulses on the intrapulse changes in P4 and LH concentrations during both preluteolysis and luteolysis.

The absence of intrapulse changes in P4 and LH concentrations in the less prominent PGFM pulse category (peak, <50 pg/mL) of the current study is compatible with the results of the **Study 2**. During a PGFM pulse with a peak > 150 pg/mL, P4 decreased between –1 h and 1 h followed by an increase at 2 h (0 h = peak of PGFM pulse), and LH increased at 2 h of the PGFM pulse. The temporal association between the LH increase and the P4 rebound within the most prominent PGFM pulses were consistent with reported intrapulse changes in spontaneous [28], E2-induced [31,33], and simulated [35,36] PGFM pulses during preluteolysis. For the PGFM pulses in the intermediate-prominence category (peak, 50–150 pg/mL), the reported increase in P4 during the ascending portion of the PGFM pulse in the Study 2 did not occur in the current study.

In conclusion, sequential treatments with 0.05 or 0.1 mg E2 initially induced a stimulatory followed by a reduced or inhibitory effect on prominence of PGFM pulses in heifers. This indicated that the chronic exposure of the uterus to elevated E2 is involved in the reduction of PGF2α secretion and may account for the reported decrease in PGFM prominence during postluteolysis. The prominence of PGFM pulses after sequential E2 treatments was greater for heifers in luteolysis than for heifers in preluteolysis. A sequential E2 treatment was associated with functional and structural luteolysis. A transient resurgence in P4 concentrations in heifers treated with sequential doses of 0.05 mg E2 was related to a less prominent PGFM pulse and a longer interval to completion of luteolysis. The LH concentration increased within 2 h after a peak of an E2-

induced PGFM pulse during preluteolysis and luteolysis and was temporally related during these periods to a complete and incomplete rebound in P4, respectively.

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CAPÍTULO 5- STUDY 4

ESTUDO ENVIADO PARA PUBLICAÇÃO

Inhibition of prostaglandin biosynthesis during postluteolysis and effects on CL regression, prolactin, and ovulation in heifers

G. Pugliesi, F.A. Khan, M.A. Hannan, M.A. Beg, G.R. Carvalho,

O.J. Ginther

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Inhibition of prostaglandin biosynthesis during postluteolysis and effects on CL regression, prolactin, and ovulation in heifers

(Inibição da biossíntese de prostaglandinas durante a pós-luteólise e seus efeitos na regressão do CL, prolactina, e ovulação em novilhas)

G. Pugliesi[a,b,c], **F.A. Khan**[a], **M.A. Hannan**[a], **M.A. Beg**[b], **G.R. Carvalho**[c], **O.J. Ginther**[a,b]

[a]*Eutheria Foundation, Cross Plains, Wisconsin 53528, Estados Unidos da América*

[b]*Department of Pathobiological Sciences, University of Wisconsin, Madison, Wisconsin 53706, Estados Unidos da América*

[c]*Departamento de Zootecnia, Universidade Federal de Viçosa,Viçosa, Minas Gerais, Brasil*

RESUMO

O início da pós-luteólise (progesterona – P4 < 1 ng/mL) foi identificado e definido como sendo 8 horas após a detecção pela ultrassonografia de 25% de redução na área (cm^2) do corpo lúteo (CL) e foi assim designado Hora 0. Flunexine meglumine (FM; n = 10) para inibir a secreção de PGF2α ou veículo (n = 9) foram administrados intramuscularmente nas Horas 0, 4, 8, 16, 24, 32, e 40. A dose de FM em cada aplicação foi de 2,5 mg/kg. Foram realizadas amostragem sanguínea e mensuração do CL e do folículo dominante a cada 8 horas iniciando no 14º dia pós-ovulação. Amostras de sangue foram também colhidas de hora em hora por 24 horas iniciando na Hora 0 para detecção dos pulsos de prolactina (PRL) e de um metabólito da PGF2α (PGFM). As concentrações nos pulsos de PGFM e PRL foram inferiores no grupo FM comparado ao grupo veículo. A concentração de PRL foi maior no pico do pulso de PGFM do que nas demais horas do pulso. Tanto a área do CL quanto a concentração de P4 não diferiram entre os grupos durante as Horas 0 a 48 (pós-luteólise). A ovulação ocorreu em nove de nove novilhas no grupo veículo

e em três de 10 novilhas do grupo FM. Os folículos anovulatórios no grupo FM cresceram até 36,2 ± 2,9 mm, e a parede folicular se apresentou espessada em decorrência de aparente luteinização. A hipótese que a PGF2α estava envolvida na redução continua da secreção de P4 e na regressão estrutural do CL durante a pós-luteólise não foi suportada. As hipóteses que os pulsos de PGFM e PRL estavam temporariamente relacionados e que o tratamento com FM sistêmico induziu a formação de folículos anovulatórios foram suportadas.
Palavras-chaves: Anovulação; Bovinos; Luteólise; PGF2α; Progesterona

ABSTRACT

The beginning of postluteolysis (progesterone < 1 ng/mL) was targeted by using 8 h after ultrasonic detection of a 25% decrease in CL area (cm^2) and was designated Hour 0. Flunixin meglumine (FM; n = 10) to inhibit PGF2α secretion or vehicle (n = 9) were given intramuscularly at Hours 0, 4, 8, 16, 24, 32, and 40. The FM was at a dose of 2.5 mng/kg at each treatment. Blood sampling and measurement of the CL and dominant follicle were done every 8 h beginning 14 d postovulation in each group. Blood samples for detection of pulses of PRL and pulses of a metabolite of PGF2α (PGFM) were obtained every hour for 24 h beginning at Hour 0. Pulse concentrations of both PGFM and PRL were lower in the FM group than in the vehicle group. Concentration of PRL was greatest at the peak of a PGFM pulse. Neither CL area (cm^2) nor progesterone concentration differed between groups during Hours 0 to 48 (postluteolysis). Ovulation occurred in nine of nine heifers in the vehicle group and in three of 10 heifers in the FM group. The anovulatory follicles in the FM group grew to 36.2 ± 2.9 mm, and the wall became thickened from apparent luteinization. The hypothesis that PGF2α was involved in the continued P4 decrease and structural CL regression during postluteolysis was not supported. The hypotheses that pulses of PGFM and PRL were temporally related and that systemic FM treatment induced an anovulatory follicle were supported.
Keywords: Anovulation; Cattle; Luteolysis; PGF2α; Progesterone

1. Introduction

Prostaglandins are involved in many reproductive processes in cattle such as luteolysis, ovulation, implantation, pregnancy, parturition, and postpartum physiology [1]. Secretion of PGF2α by the endometrium on about Day 17 (Day 0 = ovulation) is well known for its fundamental role in luteolysis [2,3]. Assay of the concentration of a PGF2α metabolite (PGFM) is often used to represent PGF2α concentration in the systemic circulation [4]. The interval between PGFM pulse peaks is about 7 h during luteolysis in cattle [5,6]. Secretion of PGF2α in pulses is a component of the luteolytic mechanism in cattle [7], as well as in sheep [8] and horses [9]. In cattle, the PGFM pulses are about 40% lower at the peak and the interpulse interval is 17% shorter during the postluteolytic period than during the luteolytic period [5,6].

For consistency among experiments, functional luteolysis in cattle is usually considered to end when progesterone (P4) decreases to 1.0 ng/mL, and postluteolysis has been defined as beginning at 0.9 ng/mL [10]. However, P4 concentration gradually declines during postluteolysis. In one study with hourly sampling, P4 decreased from 0.9 ng/mL to 0.1 ng/mL in 41 h in temporal association with a continuation of PGFM pulses [6]. It is unknown whether the continued secretion of PGF2α is required during postluteolysis either for the continued decrease in P4 concentration or the continued morphological regression of the CL.

After diminishing during postluteolysis, the secretion of prostaglandins increases late in the preovulatory period and plays a role in the ovulatory process [11,12]. Concentrations of PGF2α and PGE2 in follicular fluid increase between 24 to 30 h after the peak of the endogenous LH surge and at about the time of ovulation in cattle [13–15]. The prostaglandins apparently have an essential role in the ovulatory process in many species. Ovulation is blocked by the treatment with indomethacin, a prostaglandin inhibitor, in the doe rabbit [16], sow [17,18], ewe [19], woman [20], and mare [21] and by treatment with flunixin meglumine (FM) in the mare [22–24]. In the cow, ovulation is effectively blocked by intraovarian treatment with indomethacin [25] and intrafollicular treatment with a selective prostaglandin inhibitor, NS-398 [26]. However, the effect of systemic administration of a prostaglandin inhibitor during postluteolysis on

follicle growth and anovulation and outcome of the induced anovulatory follicle in cattle has not been reported.

The synthesis of PGF2α and PGE2 involves release of arachidonic acid (AA) from membrane phospholipids, catalyzed by the hormone-responsive enzyme cytosolic phospholipase A_2 [27]. Flunixin meglumine, one of the commonly used nonsteroidal anti-inflammatory drugs (NSAIDs), inhibits prostaglandin synthesis by competing with AA for active binding sites on the cyclooxygenase (COX) enzyme [28,29]. The COX converts AA into PGH2 that is further converted to other prostaglandins, such as PGF2α and PGE2 [29]. Treatment of heifers with FM (2.5 mg/kg body weight) every 8 h on Day 16 reduces the prominence of pulses of PGF2α during preluteolysis and luteolysis, as represented by plasma PGFM concentration in the **Study 1**.

The PGFM pulses after the beginning of luteolysis are temporally associated with an increase in PRL concentrations and with an increase in PRL pulsatility in heifers [30] and mares [31]. Synchrony (pulse peaks at same hour) of the peaks of PGFM and PRL pulses in heifers is greatest during the 12 h before the end of luteolysis and the first 12 h of postluteolysis [30]. The concentration of PRL centralized to the peak of a PGFM pulse is greatest at the peak [30]. However, whether one hormone has a direct positive effect on the other or other factors are involved in the synchrony has not been determined. The role of PRL pulses during postluteolysis and in the growth of the dominant follicle is not known, but the postluteolytic period is temporally associated with continued regression of the structure of the CL and with final development of the dominant follicle of the ovulatory follicular wave.

The purpose of the current experiment during postluteolysis in cattle was to test the following hypotheses: (1) pulses of PGF2α and PRL are temporally related, (2) PGF2α is involved in the continued functional (P4 production) regression and structural regression of the CL during postluteolysis, and (3) systemic administration of FM interferes with ovulation. Consideration was also given to the effects of inhibition of prostaglandin synthesis by systemic FM treatment on the growth of the dominant follicle.

2. Materials and methods

2.1. Animals

Dairy heifers (Holsteins) aged 23 to 29 mo and weighing 560 ± 19 kg (range: 425 to 690 kg) were used during March and April in the northern temperate zone. Heifers were selected with docile temperament and no apparent abnormalities of the reproductive tract as determined by ultrasound examination [32]. Animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research. The heifers were kept under natural light in an open shelter and outdoor paddock and were maintained by *ad libitum* access to alfalfa and grass hay, water, and trace-mineralized salt. Heifers remained healthy and in good body condition throughout the experiment. The day of ovulation (Day 0) was determined by daily transrectal ultrasound examinations [32]. If more than one CL was present or the single CL was considered undersized (< 3 cm^2) on Day 14, the heifer was not used.

2.2. Experimental design and treatments

The experimental protocol is illustrated (Fig. 1). Heifers were assigned randomly by replicate on Day 14 to a vehicle group and an FM-treated group (n = 10/group). Heifers were scanned by transrectal ultrasonography every 8 h starting at 7:00 AM on Day 14 to determine the beginning of structural luteolysis, as indicated by a decrease in the size of the CL. The hour of first detection of structural luteolysis was defined as the scan at 8-h intervals when maximum CL area (cm^2) decreased by 25% of the area on Day 14. Within 2 h after the detection of beginning of structural luteolysis, heifers were sedated as described [33]. Sedation was done only for insertion of an indwelling catheter into a jugular vein as described [34]. The first treatment was given 8 h after detection of structural luteolysis and was designated Hour 0. The 6 to 8-h delay between catheter insertion and the first treatment was used to minimize potential effects of sedation and catheterization on the experimental end points

and to allow further CL regression. It was expected that the heifers would be in late luteolysis or early postluteolysis at Hour 0. The beginning of postluteolysis was defined as the 8-h sample when P4 first decreased to < 1 ng/mL [3,35]. Heifers in the vehicle and FM groups were treated intramuscularly at Hours 0, 4, 8, 16, 24, 32, and 40. Heifers in the vehicle group were given 0.05 mL/kg of physiologic saline, and heifers in the FM group were given FM at a dose of 2.5 mg/kg at each treatment. The FM dose and interval between treatments were based on the results of the **Study 1**. Decreases in P4 concentration and CL area (cm^2) were defined as functional and structural luteolysis, respectively.

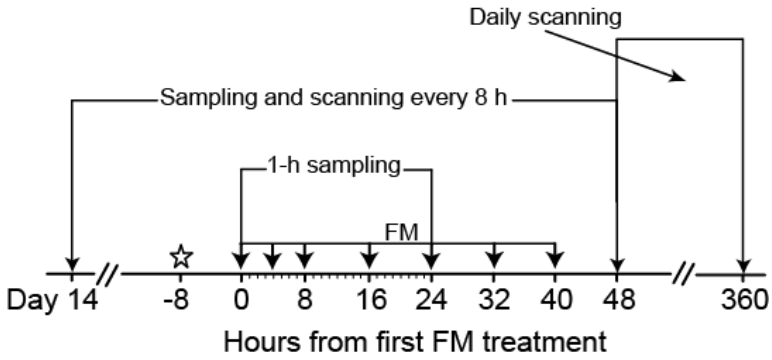

Fig. 1. Diagram of experimental design. The hour of detection of structural luteolysis is indicated by a star at Hour –8 and was designated as occurring at the 8-h scanning when maximum CL area (cm^2) had decreased to 25% of the area after the first examination 14 d postovulation. Flunixin meglumine (FM; 2.5 mg/kg body weight) was given to heifers at each indicated hour.

A blood sample (10 mL) was taken through the jugular catheter each hour for a 24-h session from Hour 0 to Hour 24 to study concentration changes in PGFM and PRL and in the number, location, and prominence of PGFM and PRL pulses. Heifers had free access to water and hay between collections of hourly samples. In addition, blood samples for P4 assay were taken from the coccygeal vein every 8 h from 7:00 AM on Day 14 until Hour 48 to determine the end of functional luteolysis in each heifer. Data analyses for the hourly sampling were limited to the heifers that were late in luteolysis or at the beginning of postluteolysis at first treatment. It was judged that the heifers in late luteolysis or early postluteolysis at the hour of treatment would be most suited for testing the hypotheses; PRL pulses are most prominent and the synchrony between PGFM and PRL pulses is greatest during the 12 h before and the 12 h after the end of luteolysis [30].

2.3. Identification of PGFM and PRL pulses

In each heifer during the 24-h session of hourly sampling, a transient hormone increase and decrease (fluctuation) encompassing at least four values in PGFM [3] or three values in PRL [30] including nadirs with a progressive increase on the ascending portion and progressive decrease on the descending portion were evaluated. When the CV of the values composing a fluctuation was at least three times greater than the mean intra-assay CV, the fluctuation was defined as a pulse [3,5,36]. These definitions were based on reports that the pulsatile episodes of PGFM [4,37] and PRL [30] during luteolysis are detectable by sampling blood at 1-h intervals. Pulses of PGFM and PRL with a peak near the beginning or end of the 24-h sampling session were included in the CV analyses only if the peak was preceded or followed, respectively, by at least one lower value, but the lower value was not used to calculate nadir concentrations. Nadirs 1 and 2 were defined as the nadirs at the beginning and end of a pulse, respectively. When two identified PGFM peaks were separated by only one hour, the two peaks were considered part of the same pulse, and the peak with the greatest concentration was used in the analyses; double peaks occurred in 13% of PGFM pulses.

Synchrony between PGFM and PRL pulses was defined as the occurrence of the peak of CV-identified pulses of both PGFM and PRL at the same hour. Pulses of PGFM were partitioned into nonprominent and prominent pulses based on pulses with peaks of <50 pg/mL and ≥ 50 pg/mL, respectively, owing to the report that nonprominent pulses in the Study 3 were ineffective. The prominence of PRL pulses was also considered, using pulses with peaks of < 10 ng/mL and ≥ 10 ng/mL to represent nonprominent and prominent pulses, respectively, as reported [30]. Pulse prominence was considered in the temporal relationship between the concentrations of PRL during a PGFM pulse and concentrations of PGFM during a PRL pulse.

2.4 Ultrasound scanning

The ovaries were scanned by transrectal ultrasound every 8 h beginning on Day 14 until Hour 48 (Hour 0 = first treatment) and then daily until Hour 360 if ovulation did not occur. A duplex B-mode (gray-scale) and pulse-wave color Doppler ultrasound instrument (Aloka SSD 3500; Aloka America, Wallingford, CT) equipped with a 7.5-MHz linear transducer was used for the scanning. The diameter of the four largest follicles and the maximum CL area (cm^2) were recorded. The four largest follicles were used so that the growth of the one follicle among the four that became the preovulatory follicle could be assessed. The maximum CL area was determined using a B-mode still image and the tracing function. For CL with an anechoic fluid-filled cavity, the area of the cavity was subtracted from the total area [38,39]. Anovulation was defined as failure of ovulation of a dominant follicle that reached 15–17 mm and then maintained or increased in diameter for ≥ 5 d. Luteinization of an anovulatory follicle was assumed by thickening and increased echogenicity of the follicular wall by visual evaluation on the gray-scale image as described [23]. The presence of blood flow in the luteinized wall of an anovulatory follicle was detected by color-Doppler signals as described [40]. The gray-scale and color-Doppler observations were used only for preliminary information and were not analyzed critically.

2.5 End points

Comparisons between groups for the concentrations of PGFM and PRL were made during the 24-h session of hourly sampling. End points for the CV-identified PGFM and PRL pulses were the overall mean for all values in a pulse and area under curve of a pulse and concentrations at pulse events (peak, Nadir 1, Nadir 2, amplitude). Comparisons between groups were made of the intervals between Nadir 1 and Nadir 2 of a pulse, the interval from peak to peak and Nadir 2 to Nadir 1 of adjacent pulses, the number of PGFM and PRL pulses during the 24-h hourly sampling session, and the frequency of synchrony between PGFM and PRL pulses (synchrony = both pulses peak at same hour). The number of PGFM pulses/session was compared to the number of PRL

pulses for each treatment group. The concentration profile of PRL during a PGFM pulse and concentration of PGFM during a PRL pulse were determined. Thus, the frequency of pulse synchrony and concentrations of one hormone relative to the other used either the PGFM pulse or the PRL pulse as the reference pulse.

In addition to the PGFM and PRL end points, P4 concentration, CL area (cm^2) at the 8-h scanning intervals for Hours 0 to 48, and the diameter of the dominant follicle from Hour 0 to ovulation or Hour 96 were compared between the two groups. Comparisons also were made between groups for the interval from first treatment to ovulation, the number of heifers in which the dominant follicle ovulated or did not ovulate, and the number of anovulatory follicles that apparently became luteinized based on increased thickening of the wall of the anovulatory follicle.

2.6. Hormone assays

The blood samples were immediately placed in ice water for 10 min before centrifuging (2000 X *g* for 10 min). The plasma was decanted and stored (–20 ºC) until assay. The plasma samples were assayed for PGFM by an ELISA that was developed and validated in our laboratory for use with bovine plasma [5]. Plasma concentrations of PRL were determined by an RIA that was described and validated for use in bovine plasma in our laboratory [43]. Plasma P4 concentrations were assayed with a solid-phase RIA kit containing antibody-coated tubes and I^{125}-labeled P4 (Coat-A-Count Progesterone, Diagnostic Products Corporation, Los Angeles, CA, USA) as validated and described [3]. The intra- and interassay CV and sensitivity, respectively, were 10.1 %, 9.8 %, and 13.7 pg/mL for PGFM and 10.0 %, 3.9 %, and 0.39 ng/mL for PRL. The intra-assay and sensitivity for P4, respectively, were 8.7 %, and 0.02 ng/mL.

2.7. Statistical analysis

Data were examined for normality using the Shapiro-Wilk test. Data that were not normally distributed were transformed to natural logarithms, ranks, or square roots. Hourly concentrations of PGFM and PRL and the P4, CL, and

follicle end points at 8-h intervals were analyzed for the main effects of group and hour and the interaction of group by hour. For analyses involving pulse prominence, a three-way factorial was used with group, hour, and prominence as factors. The SAS PROC MIXED procedure (Version 9.2; SAS Institute) was used for analysis with a REPEATED statement to account for the autocorrelation between sequential measurements. The Least Significant Difference (LSD) test was used for comparisons between hours within a group. Discrete or single-point data for characteristics and intervals for PGFM and PRL pulses were analyzed for differences between groups by Student's unpaired *t*-test. The LSD test was used to locate differences among hours within each group. Student's unpaired *t*-test was used to locate differences between follicle diameters for each hour. Fisher's exact test and chi-square distribution were used for comparisons of frequency data. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $P > 0.05$ to $P \leq 0.1$ indicated that significance was approached. Data are presented as the mean ± SEM, unless otherwise indicated.

3. Results

Probabilities for a group effect, hour effect, and a group-by-hour interaction for the factorial analyses are given in the figures and the probabilities for differences in discrete end points are given in the table or text. Concentrations of P4 at 8-h intervals indicated that one heifer in the vehicle group was in preluteolysis at the time of the first treatment and was not used in the analyses. In the remaining 19 heifers, a 25% reduction in CL area (cm^2) occurred on Day 18.0 ± 0.6 (Fig. 2). Eight hours after the 25% reduction in CL area (hour of first treatment; Hour 0) the P4 concentration was < 1 ng/mL in 15 heifers, indicating they were in postluteolysis. In the remaining four heifers, concentration was > 1 ng/mL at Hour 0 (1.2–2.8 ng/mL). The mean P4 concentration was 0.8 ± 0.1 ng/mL at Hour 0 and 0.2 ± 0.03 ng/mL at Hour 48. Therefore, all 19 heifers (9 and 10 in the vehicle and FM groups, respectively) were used in the analysis without subgrouping into heifers that were in luteolysis versus postluteolysis at Hour 0. Both CL area and P4 concentration

decreased progressively from Hours 0 to 48 (hour effect) with no significant difference between the vehicle and FM groups or an interaction in CL area (cm^2) or in P4 concentration during the 8-h intervals from Hours 0 to 48 (Fig. 2).

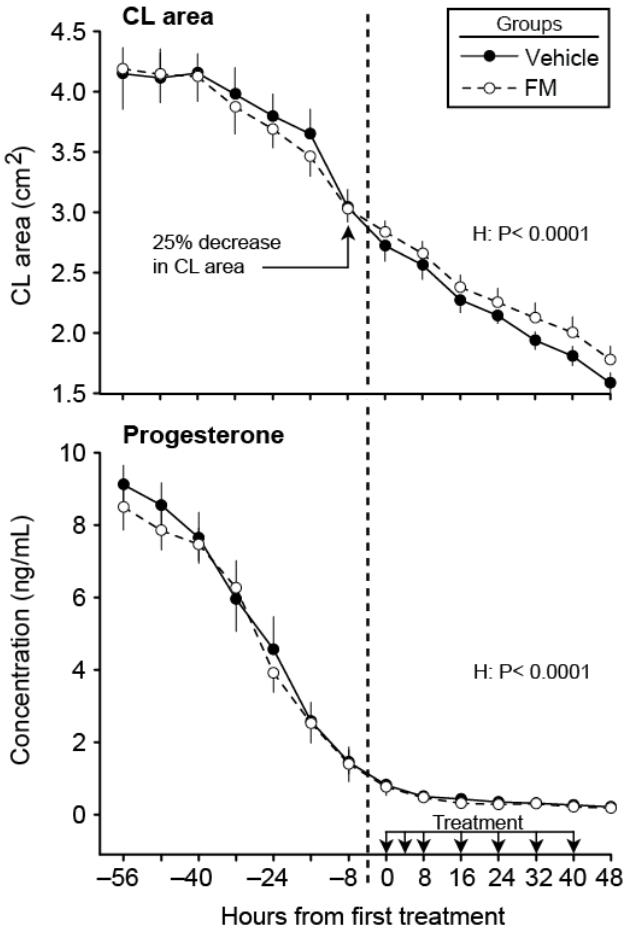

Fig. 2. Mean ± SEM CL area (cm^2) and progesterone concentrations in a vehicle group (n = 9) and an FM group (n = 10) treated at Hours 0, 4, 8, 16, 24, 32, and 40 (Hour 0 = 8 h after detection of structural luteolysis as indicated). The factorial analysis was done from Hours 0 to 48. Main effect of Hour (H) is shown. There was not a main effect of group or a group by hour interaction for either end point.

Concentration of PGFM during the 24 h of hourly sampling showed significant group and hour effects and an interaction that approached significance (Fig. 3). The group effect represented a lower mean PGFM concentration in the FM group (23.2 ± 3.1 pg/mL) than in the vehicle group (40.7 ± 4.4 pg/mL). The tendency for an interaction was attributable primarily to significantly reduced concentrations in the FM group during Hours 6 to14, 17, and 22 to 24. There were fewer CV-identified pulses of PGFM/heifer during a 24-h session in the FM group than in the vehicle group (Table 1). The PGFM

pulses showed a group effect from lower overall concentration in the FM group than in the vehicle group (Fig. 3, Table 1). The hour effect was significant as expected. An interaction represented in part lower concentration in the FM group at –1, 1, and 2 h (0 h = peak). Most discrete or single-point characteristics of PGFM pulses were similar between groups, but the concentration at Nadir 2 was less and the area under the curve tended to be less in the FM group (Table 1). If only three values for a PGFM pulse had been accepted for pulse identification, additional pulses of PGFM would have been detected by the CV method in only 1 of 9 heifers in the vehicle group compared to 7 of 10 heifers in the FM group ($P < 0.02$).

Table 1. Mean ± SEM for discrete end points during PGFM and PRL pulses in heifers treated with vehicle (n = 9) and Flunixin Meglumine (FM; n = 10).

	PGFM			PRL		
Pulse characteristic	Vehicle	FM	Probability	Vehicle	FM	Probability
Number of pulses	24	7	---	44	33	---
Number of pulses/24h	2.7 ± 0.4	0.7 ± 0.2	P < 0.0001	4.9 ± 0.3	3.3 ± 0.3	P < 0.0002
PGFM (pg/mL); PRL (ng/mL)						
Overall [a]	48.5 ± 6.8	26.5 ± 4.9	P < 0.03	11.8 ± 1.1	8.6 ± 0.7	NS
At Nadir 1[b]	19.6 ± 3.1	13.1 ± 5.4	NS	6.5 ± 1.1	4.5 ± 0.7	P < 0.07
At peak	105.1 ± 26.9	59.1 ± 16.7	NS	23.7 ± 2.4	16.3 ± 2.3	P < 0.04
At Nadir 2[b]	14.8 ± 1.8	7.3 ± 1.5	P < 0.02	7.8 ± 1.5	5.1 ± 0.6	P < 0.05
Amplitude[c]	82.0 ± 28.1	46.0 ± 17.0	NS	18.1 ± 3.2	12.3 ± 2.3	P < 0.08
Area under curve[d]	100.5 ± 33.4	50.9 ± 19.1	P < 0.1	19.8 ± 3.2	11.9 ± 2.6	P < 0.04
Intervals (h)						
Nadir 1 to Nadir 2[b]	3.5 ± 0.2	4.6 ± 0.4	P < 0.01	4.8 ± 0.4	4.0 ± 0.7	NS
Nadir 1 to peak[b]	2.2 ± 0.2	2.3 ± 0.5	NS	1.7 ± 0.2	2.4 ± 0.3	P < 0.01
Peak to Nadir 2[b]	2.4 ± 0.3	1.7 ± 0.3	P < 0.1	1.8 ± 0.2	2.3 ± 0.3	P < 0.07
Peak to peak	6.1 ± 0.7	---	---	3.7 ± 0.3	5.0 ± 0.6	P < 0.02
Nadir 2 to Nadir 1[b]	1.0 ± 0.4	---	---	0.4 ± 0.2	1.0 ± 0.5	P < 0.1

[a] Overall refers to the average of all values in the pulse.

[b] Nadir 1 and Nadir 2 are at the beginning and end of a pulse, respectively. The interval from Nadir 2 to Nadir 1 refers to adjacent pulses.

[c] Amplitude is the concentration at the peak minus the concentration at Nadir 1.

[d] Area under the curve (ng/mL h) of a pulse was assessed for –1, 0, and 1 h.

NS= not significant.

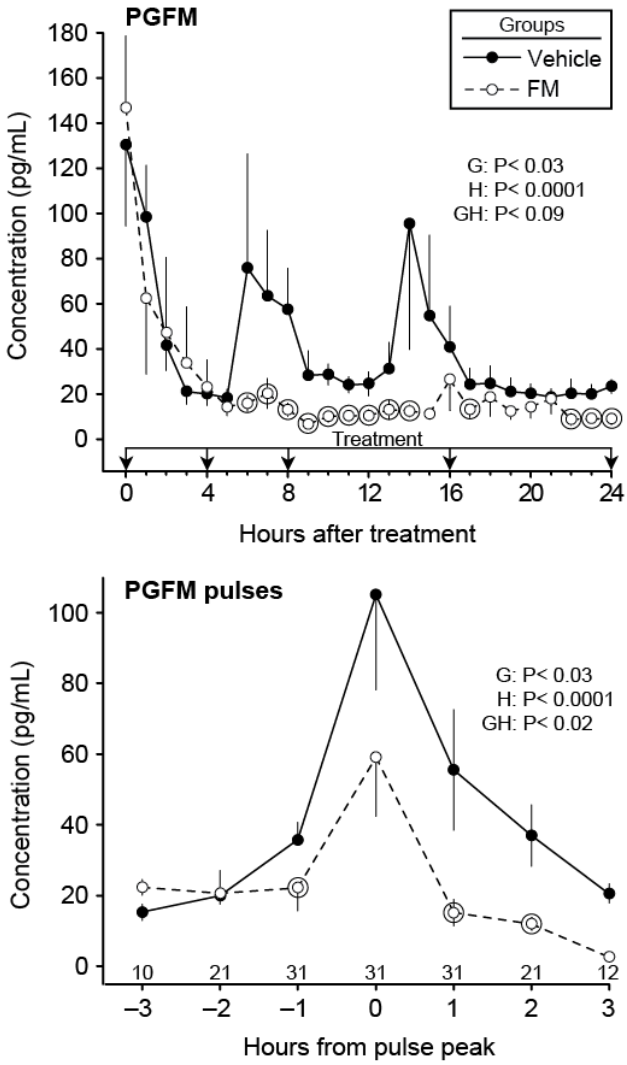

Fig. 3. Mean ± SEM hourly concentrations of PGFM and during PGFM pulses in a vehicle group (n = 9) and FM-treated group (n = 10). The combined number of observations for pulses are shown above the hour scale for each hour of a pulse. Main effects of group (G) and hour (H) and the interaction (GH) are shown. A circle around an FM mean indicates a difference (P < 0.05) from the vehicle mean at that hour.

The hourly PRL concentrations had only an hour effect, reflecting transient increases (P ≤ 0.05) over Hours 5 to 13 and Hours 15 to 21 (Fig. 4). There were fewer CV-identified PRL pulses/heifer in the FM group than in the vehicle group (Table 1). Although the factorial analysis of pulses showed only the expected hour effect (Fig. 4), the discrete data indicated that area under the curve and concentration at the pulse peak and at Nadir 2 were significantly less in the FM group than in the vehicle group (Table 1). Nadir 1 and amplitude of PRL pulses approached lower concentrations in the FM group. The intervals between all PRL pulse events were greater or approached being greater in the FM group

than in the vehicle group. In each of the vehicle and FM groups, there were more (P < 0.0001) PRL pulses than PGFM pulses (Table 1).

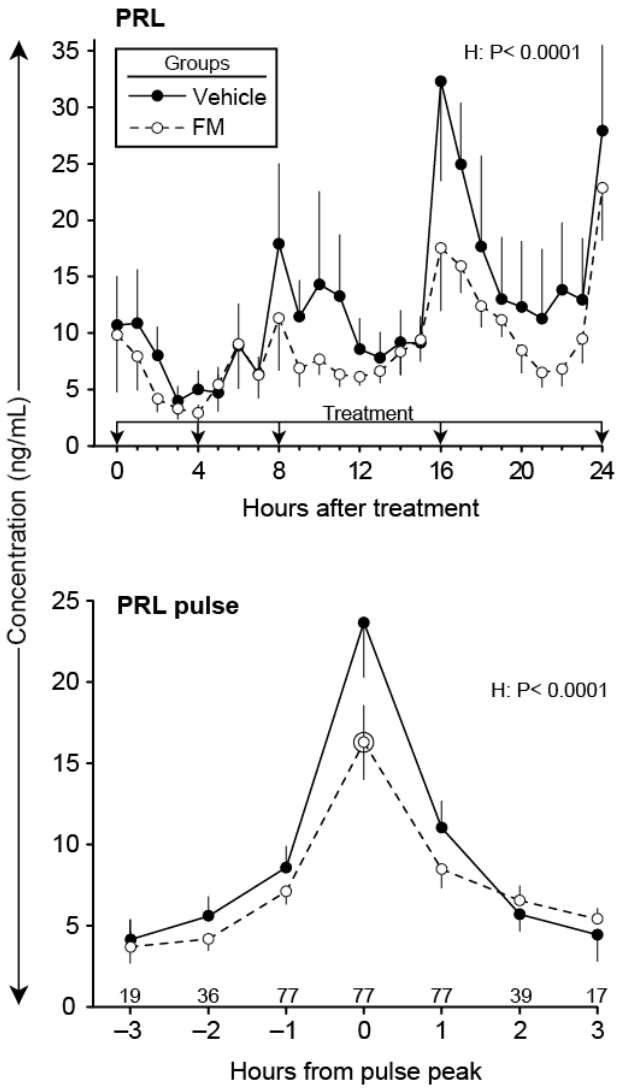

Fig. 4. Mean ± SEM hourly concentrations of PRL and during PRL pulses in a vehicle group (n = 9) and FM-treated group (n = 10). The combined number of observations for pulses are shown above the hour scale for each hour of a pulse. The main effect of hour (H) is shown for each panel. A circle around an FM mean indicates a difference (P < 0.05) from the vehicle mean at that hour.

During a PGFM pulse with a peak (0 h) of < 50 vs. ≥ 50 pg/mL representing nonprominent vs. prominent pulses, respectively, the associated concentrations of PRL showed only an interaction of prominence (< 50 vs. ≥ 50 pg/mL) and hour (Fig. 5). The interaction represented an increase in PRL concentrations in each of the vehicle and FM groups between –1 and 0 h and a decrease between 0 and 1 h for the prominent PGFM pulses. There were no

changes in the nonprominent PGFM pulses. The effect of treatment group and all interactions involving group were not significant. The PRL concentration during prominent PGFM pulses combined for vehicle and FM groups (24.2 ± 6.2 ng/mL) was greater (P < 0.02) than at the combined nonprominent PGFM pulses (9.1 ± 2.0 ng/mL).

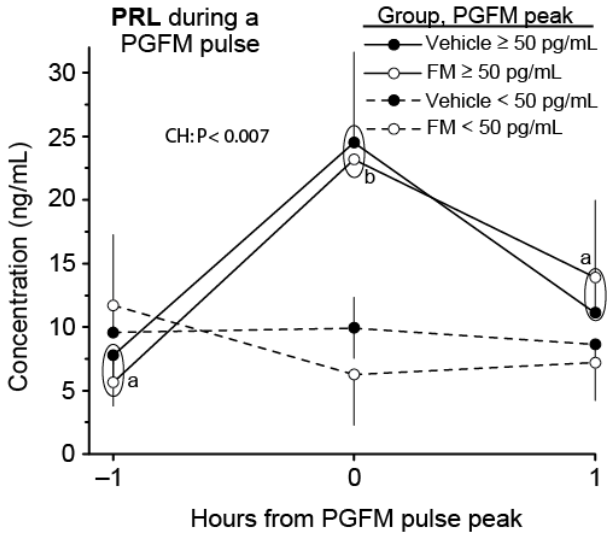

Fig. 5. Mean ± SEM for concentrations of PRL within the hours of a PGFM pulse centralized to the PGFM peak in the vehicle group and the FM-treated group. The PGFM pulses are partitioned into nonprominent (peak, < 50 pg/mL) and prominent (peak, ≥ 50 pg/mL) within each group. The number of pulses for each group and prominence were 13, 4, 11, and 3 as listed from top to bottom. In the factorial analysis, only the interaction of prominence and hour (PH) was significant. ab = circled means that were different (P < 0.05) among hours within the vehicle group with prominent pulses and within the FM group with prominent pulses.

In addition, the area under the PRL curve (ng/mL h) was greater (P < 0.05) in each group when associated with a prominent PGFM pulse (vehicle, 19.6 ± 6.1; FM, 21.8 ± 18.4) than when associated with a nonprominent pulse (vehicle, 6.4 ± 1.5; FM, 5.8 ± 2.2). Combined for both groups, the peak of a prominent PGFM pulse was synchronized (same hour) with the peak of a prominent PRL pulse (≥ 10 ng/mL) in 8/17 (47%) pulses. In contrast, the peak of a prominent PRL pulse was synchronized with the peak of a prominent PGFM pulse in 8/50 (16%) prominent PRL pulses (8 of 17 vs. 8 of 50, P < 0.01).

The concentrations of PGFM centralized to the peak of a nonprominent PRL pulse (< 10 ng/mL) and to the peak of a prominent pulse (≥ 10 ng/mL) are shown (Fig. 4). At –1 to 1 h of a PRL pulse, the associated concentrations of PGFM showed a group effect (vehicle, 48.3 ± 7.7 pg/mL; FM, 16.4 ± 2.1 pg/mL) and an hour effect (–1 h, 28.2 ± 5.3 ng/mLa; 0 h, 45.0 ± 10.4 ng/mLb; 1 h, 28.2

± 6.3 ng/mLa); means with different letters are different (P < 0.05). The prominence effect was not significant, and the only significant interaction was for prominence by hour. Significant changes in PGFM concentrations among hours were found only in the vehicle group with a prominent PRL pulse (≥ 10 ng/mL) as shown (Fig. 6).

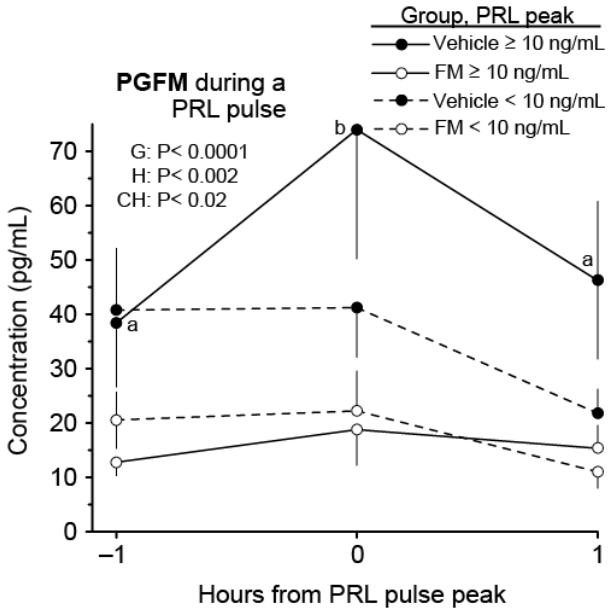

Fig. 6. Mean ± SEM for concentrations of PGFM within the hours of a PRL pulse centralized to the PRL peak in the vehicle group and the FM-treated group. The PRL pulses are partitioned into nonprominent (peak, < 10 ng/mL) and prominent (peak, ≥ 10 ng/mL) within each group. The number of pulses for each group and prominence were 29, 21, 15, and 12 as listed from top to bottom. The main effect of group (G) and hour (H) are shown. The only significant interaction was prominence by hour (PH). ab = means within the vehicle group with prominent pulses with different letters are different (P < 0.05) among hours.

At least one PGFM pulse was detected in each heifer in the vehicle group. In the FM group, PGFM pulses were detected in six heifers, and no pulses were detected in four heifers (0 of 9 vs. 4 of 10; P < 0.05). Analysis of these two FM subgroups for the hourly concentration of PRL during the 24-h session showed subgroup and hour effects (Fig. 7). The subgroup effect represented greater overall PRL concentration in the heifers with PGFM pulses (30.2 ± 4.6 ng/mL) than in the heifers with no PGFM pulses (12.8 ± 3.4 ng/mL). Although the interaction was not significant, PRL concentrations were lower in the heifers with no detected PGFM pulses at Hours 5–8 and 13.

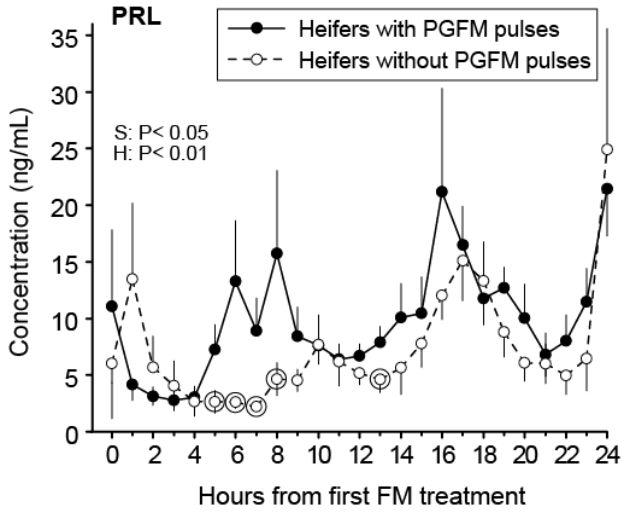

Fig. 7. Mean ± SEM for concentrations during hourly blood sampling of PRL in heifers of the FM group with PGFM pulses (n = 6) and without PGFM pulses (n = 4). Main effects of subgroup (S) and hour (H) are shown. A circle around a subgroup mean for heifers without PGFM pulses indicates a difference (P < 0.05) from the mean for the other subgroup.

The diameter of the dominant follicle for Hours 0 to 96 showed an hour effect and an interaction of group by hour (Fig. 8). The interaction represented greater diameter of the dominant follicle in the FM group at Hour 72 (approached significance; P ≤ 0.1) and at Hour 96 (P < 0.05). The number of heifers that ovulated was greater (P < 0.0004) in the vehicle group (9 of 9) than in the FM group (3 of 10). For the heifers that ovulated, the interval from Hour 40 (last treatment) to ovulation was not different between the vehicle group (61.3 ± 12.5 h) and the FM group (48.0 ± 8.0 h). The increasing diameter of the seven anovulatory follicles in the FM group is shown (Fig. 8). The anovulatory follicles reached a maximum diameter of 36.2 ± 2.9 mm. Two of seven anovulatory follicles collapsed during scanning at Hours 144 and 148. The remaining anovulatory follicles (n = 5) collapsed or began to regress 10 d after the first treatment. It was estimated that gray-scale indicators of luteinization of the follicle wall (increased thickness and echogenicity) of the anovulatory follicles began at Hour 92 ± 13. Color-Doppler signals of blood flow were detected in all luteinized follicles.

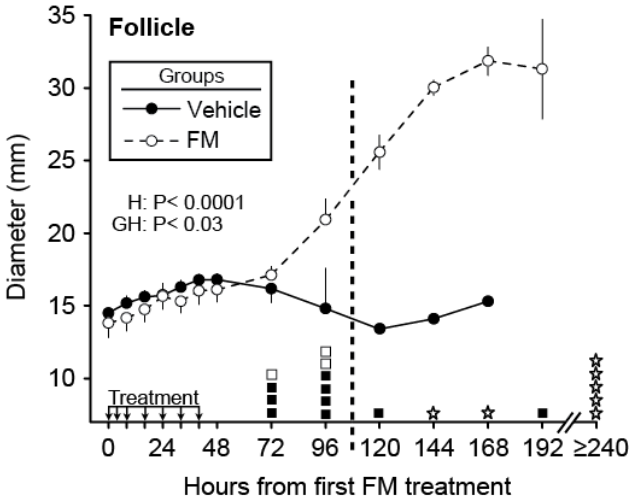

Fig. 8. Mean ± SEM diameter of the dominant follicle in a vehicle group (n = 9) and an FM group (n = 10) treated at Hours 0, 4, 8, 16, 24, 32, and 40 (Hour 0 = 8 h after detection of structural luteolysis). The factorial analysis was done from Hours 0 to 96. Main effect of hour (H) and interaction of group by hour (GH) are shown. The squares above the hour scale represent the hours of ovulation in individuals of the vehicle group (solid squares) and FM group (open squares). The stars represent the hour of detection of collapse or regression of the luteinized anovulatory follicle in individuals in the FM group.

4. Discussion

The technique of using an ultrasonically detected 25% reduction in CL area (cm^2) and beginning the treatments 8 h later for targeting late luteolysis and postluteolysis for beginning the treatment challenges was successful in 95% of heifers. Most heifers (79%) were in postluteolysis (P4 < 1 ng/mL) at the first treatment (Hour 0). The remaining 21% were in the later portion of functional luteolysis (P4 mean, 1.7 ng/mL). For future experiments, beginning treatment at the 8-h interval with 25% and 12.5% in CL reduction would have resulted in 50% and 60 %, respectively, of heifers being in luteolysis.

The PGFM concentrations and pulsatility during postluteolysis in the dairy heifers in the vehicle group seemed similar to those of previous reports [5,42]. Treatment with FM during postluteolysis in the current study effectively reduced the concentrations and pulses of PGFM. This was indicated in the FM group by the approximately two-fold reduction in each of the mean hourly concentrations, overall pulse concentrations, and area under the curve of PGFM pulses and a four-fold reduction in number of pulses. These results indicated that the effect of FM treatment on reduction in PGFM concentrations and pulses in heifers during postluteolysis is similar to the reported effects

during preluteolysis and luteolysis with the same FM dose in the Study 1. The PGFM concentrations in the current study in the FM group were reduced to below baseline levels as indicated by hourly concentrations for most hours and by the nadirs of PGFM pulses.

Hypothesis 1— that pulses of PGF2α and PRL are temporally related during postluteolysis— was supported. This was indicated by: (1) the reduction in PGFM concentrations and pulses in the FM group was associated with a reduction in number of pulses of PRL, reduced area under the curve of PRL pulses, and the significant or approaching significant decrease in PRL concentrations at the components of the PRL pulses; (2) increased concentration of PRL at the peak and area under the curve when centralized to the peak of a prominent PGFM pulse (≥ 50 pg/mL) in the vehicle and FM groups; (3) reduced PRL concentrations in the FM group in heifers that did not have PGFM pulses; and (4) considerable synchrony (47%; peaks at same hour) of a prominent PGFM pulse (≥ 50 pg/mL) with a prominent PRL pulse (≥ 10 ng/mL). The partitioning of PGFM pulses according to nonprominence and prominence was justified by the increase in PRL centralized to a PGFM pulse peak only when the PGFM pulses were prominent.

The reduction in number of PRL pulses in the FM group was consistent with the increased peak-to-peak interval and the wider base (Nadir 1 to Nadir 2) of a PRL pulse. The increase in PGFM concentrations during the hours of a prominent PRL pulse in the vehicle group but not in the FM group may have been related to inhibition of PGF2α biosynthesis in the FM group. More PRL pulses than PGFM pulses in the vehicle group cannot be attributed to the CV-identification requirement that four values were used to detect PGFM pulses, whereas three values were used to detect PRL pulses. Only 1 of 9 heifers in the vehicle group had additional PGFM pulses that would have been identified if only three values were required. The lower frequency of synchrony between PGFM and PRL pulses when a PRL pulse was the reference pulse was a reflection of the greater number of PRL than PGFM pulses. That is, more PRL pulses than PGFM pulses necessitated that many PRL pulses would not be synchronized with a PGFM pulse.

It is unknown whether the temporal relationships between PGFM and PRL pulses represented a positive effect of one on the other or whether other

factors are involved. In this regard, an increase in estradiol occurs during luteolysis [43] and within the hours of a PGFM pulse [42]. The prominence of estradiol-induced PGFM pulses increases with increasing estradiol doses as observed in the Study 2. Furthermore, concentration of PRL increases before estrus in sheep and rats and is thought to be associated with the increase in estradiol, as indicated by the increase in concentration of PRL after estradiol administration [44]. Secretion of PRL is also stimulated by estradiol in bovine pituitary-cell cultures [45]. Further study will be required to determine if PGF2α affects PRL or PRL affects PGF2α and to clarify the role of other factors in PGF2α and PRL pulse synchrony.

Hypothesis 2— that PGF2α is utilized in the continued functional and structural regression of the CL during postluteolysis— was not supported. The decrease in P4 concentration and in CL area (cm^2) did not differ between groups during the 48 h of postluteolysis; both end points decreased progressively during the 48 h and therefore the hypothesis was adequately considered. During Hours 0 to 48, mean P4 concentration decreased from 0.8 to 0.2 ng/mL. Nine heifers reached 0.1 ng/mL by Hour 48. A previous preliminary conclusion indicated that P4 decreases from 0.9 to 0.1 ng/mL in 41 h [6] which seems close to the current results. The reported time required for the completion of luteolysis to 1 ng/mL is at least partly dependent on the prominence of PGF2α secretion as represented by PGFM concentrations in the **Studies 1 and 3**.

The reduction in both PGFM and PRL by FM treatment precluded distinguishing between the effects of PGF2α and PRL. However, in the current study during postluteolysis, the reduction in both PGFM and PRL in the FM group indicated that neither circulatory PGF2α nor PRL were involved in the continued functional and structural CL regression after P4 decreased to < 1 ng/mL. A reservation is that residual PGFM or PRL after the FM treatment may have had an effect. A literature search did not disclose compelling indications that PRL has a luteal role during or after luteolysis in cattle. However, the findings that the PRL gene is expressed in luteal cells and PRL protein is present in the smooth muscle of the intraluteal arterioles and endothelial cells are at least compatible with a role of PRL in luteolysis in cattle [46]. The negative results in the current study did not clarify whether the continued

functional and structural CL regression after P4 reaches < 1 ng/mL is a carry-over effect of the previously active luteolytic mechanism or whether an active mechanism continues to be involved. In this regard, the apoptosis process in the steroidogenic and accessory cells of the CL is mediated by local factors such as endothelin-1, cytokines, vasoactive peptides, and nitric oxide [47–49].

The reduction in PGF2α and PRL during Hours 0–48 did not alter the growth of the dominant follicle. The follicle increased in the combined treated and vehicle groups from a mean of 13.5 mm at Hour 0 to 16.5 mm at Hour 48 (8 h after last treatment). This finding represents the first reported indication that PRL is not a requirement for growth of the preovulatory follicle during postluteolysis. Ovulation in the 12 heifers that ovulated occurred between Hours 48 and 72 in four heifers and between Hours 72 and 96 in six heifers. Therefore, it is assumed that anovulation in the FM group began primarily between Hours 48 and 96 or within 2 d after the last FM treatment. The precise temporal relationship between the last treatment of FM and anovulation and the associated concentrations of PGFM and PRL were not determined. However, the half-life of FM after the last treatment increases substantially in cattle when repeated treatments are given; FM half-life in cattle has been reported as 4 h after a single treatment of 2.2 mg/kg and 26 h after four daily treatments [50]. Most likely, therefore, the continued effects of FM approached the expected time of ovulation. However, specific study with frequent sampling of blood and frequent scanning for ovulation is indicated.

Hypothesis 3— that systemic administration of FM interferes with ovulation— was supported. Repeated intramuscular FM treatment of 2.5 mg/kg for 2 d after the end of luteolysis efficiently blocked ovulation as indicated by fewer heifers ovulating in the FM group (3 of 10) than in the vehicle group (9 of 9). The ovulation blockage by FM likely involved inhibition of the COX activity and consequently prostaglandin biosynthesis. In this regard, the local increase in concentration of prostaglandins induces vasodilatation in the follicle blood vessels, augments vascular permeability, and stimulates the degradation of extracellular matrix that are involved in the follicle rupture [19,51]. In mares, repeated systemic FM treatment also effectively blocks ovulation, leading to the development of the syndrome of hemorrhagic anovulatory follicle [22–24]. In cattle, the inhibition of prostaglandins after the LH surge by intraovarian

treatment with indomethacin [25] and intrafollicular treatment with a selective COX-2 inhibitor (NS-398 [26]) effectively blocks ovulation. However, the intramuscular or intrauterine treatment with indomethacin apparently did not sufficiently inhibit local prostaglandin secretion [25]. The efficacy of the intramuscular FM treatment in the current study used a less invasive and more practical experimental approach for blocking ovulation in cattle, compared to the intraovarian or intrafollicular treatment previously reported [25,26].

The diameter of the dominant anovulatory follicle in the FM group increased after the expected time of ovulation (Hours 72 and 96) and continued to increase to an average of 36 mm and was > 40 mm in three heifers. The initiation of luteinization of the anovulatory follicle wall seemed to be near the expected time of ovulation, indicating that the inhibition of PGs blocked ovulation but did not affect the LH-induced luteinization of the follicular wall. The presence of an anovulatory follicle was observed for ≥ 10 d in five out of seven heifers. The color-Doppler signals of blood flow in the luteinized wall indicated that this area was functional and most likely secreting P4, but the P4 concentration was not determined. In this regard, reported circulating P4 concentration in heifers with induced luteinized anovulatory follicles was similar to concentration in heifers with a spontaneous CL [26].

The ultrasonography of FM-induced luteinized anovulatory follicles resembled those reported for spontaneous luteinized cystic follicles in cattle [52–55]. Cystic anovulatory follicles cause economic loss in the diary industry [52,53,55]. The endocrine and cellular changes after the formation of cysts have been characterized (reviewed in [53]), but the cellular and molecular events that take place prior to the development of cysts remain poorly understood [56]. The similar characteristics of anovulatory follicles in the FM-treated heifers in the current study and in spontaneous cystic follicles in reported studies [52–54] indicate that systemic FM treatment may be useful for study of the etiology, pathology, and treatment of anovulatory cysts.

In conclusion, repeated treatment of heifers with FM beginning near the mean onset of postluteolysis (mean, P4 < 0.8 ng/mL) reduced the concentrations and number of pulses of PGFM and PRL. The reduced PGF2α and PRL did not alter the concentrations of P4 or CL area (cm^2) or growth of the

dominant follicle during postluteolysis (48 h after the first FM treatment). The FM treatment blocked ovulation in 70% of heifers.

Acknowledgments

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CONCLUSÕES GERAIS

Em conjunto pode-se concluir por estes estudos que:

Baseado nas concentrações de PGFM, a secreção de PGF2α durante os períodos pré-luteolítico, luteolítico e pós-luteolítico é eficientemente inibida pelo tratamento com 2,5 mg/kg de flunexine meglumine (FM), porém o grau de eficiência do FM pode variar de acordo com a fase da luteólise.

A concentração de E2 pode exercer um efeito bifásico na regulação do mecanismo endometrial envolvido com a síntese de PGF2α antes, durante e após a luteólise. Durante a pré-luteólise a proeminência dos pulsos de PGFM aumenta de acordo com o aumento da dose de E2 administrado, entretanto, após a indução da luteólise a elevação crônica do E2 pode estar envolvida com o mecanismo de redução da secreção de PGF2α no período pós-luteolítico.

A redução da proeminência do pulsos de PGFM no período luteolítico através da inibição da secreção de PGF2α pelo tratamento com FM ou pela elevação crônica do E2 está associado à maior duração da luteólise funcional, podendo resultar até em resurgimento transitório das concentrações de P4.

Pela primeira vez foi reportado que as mudanças das concentrações de P4 e LH e a alteração do fluxo sanguíneo luteal, durante um pulso de PGFM, são dependentes da proeminência (pico e amplitude) deste pulso.

O tratamento sequencial (três doses) ou com uma única dose de 0,05 ou 0,1 mg de E2 no período pré-luteolítico induz precocemente a luteólise funcional e estrutural em novilhas.

As concentrações e os pulsos proeminentes de PGFM e PRL são temporariamente associados no início do período pós-luteolítico.

A inibição da secreção de PGF2α pelo FM durante a pós-luteólise não afeta a redução de P4 e da área luteal que ocorrem durante a luteólise estrutural, assim como não altera o crescimento do folículo dominante nas primeiras 48 horas após o início do tratamento, entretanto, bloqueia a ovulação e induz a formação de folículos luteinizados anovulatórios em 70% das novilhas tratadas.